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(54) Title: SUBSTRATE TRAPPING PROTEIN TYROSINE PHOSPHATASES

(57) Abstract

Novel protein tyrosine phosphatases in which the invariant aspartate residue is replaced with an alanine residue and which bind to a tyrosine phosphorylated substrate and are catalytically attenuated are described. Also described are methods of identifying tyrosine phosphorylated proteins which complex with the described protein tyrosine phosphatases.

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SUBSTRATE TRAPPING PROTEIN TYROSINE PHOSPHATASES

Background of the Invention

The protein tyrosine phosphatase (PTP) family of
enzymes consists of more than 500 structurally diverse
5   proteins which have in common the highly conserved 250
amino acid PTP catalytic domain, but which display
considerable variation in their non-catalytic segments
(Charbonneau and Tonks, Annu. Rev. Cell Biol. 8:463-493
(1992); Tonks, Semin. Cell Biol. 4:373-453 (1993)). This
10 structural diversity presumably reflects the diversity of
physiological roles of individual PTP family members, which
in certain cases have been demonstrated to have specific
functions in growth, development and differentiation (Desai
et al., Cell 84:599-609 (1996); Kishihara et al., Cell
15 74:143-156 (1993); Perkins et al., Cell 70:225-236 (1992);
Pingel and Thomas, Cell 58:1055-1065 (1989); Schultz et
al., Cell 73:1445-1454 (1993)). Although recent studies
have also generated considerable information regarding the
structure, expression and regulation of PTPs, the nature of
20 the tyrosine phosphorylated substrates through which the
PTPs exert their effects remains to be determined. Studies
with a limited number of synthetic phosphopeptide
substrates have demonstrated some differences in substrate
selectivity of different PTPs (Cho et al., Protein Sci. 2:
25 977-984 (1993); Dechert et al., Eur. J. Biochem. 231:673-
681 (1995)), and have indicated preferences for certain
amino acid residues at particular positions around the
phosphorylated tyrosine residue (Ruzzene et al., Eur. J.
Biochem. 211:289-295 (1993); Zhang et al., Biochemistry
30 33:2285-2290 (1994)). This indicates that PTPs display a
certain level of substrate selectivity in vitro, although

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the physiological relevance of the substrates used in these studies is unclear.

Summary of the Invention

As described herein, the substrate specificity of mammalian protein tyrosine phosphatases (PTPs) has been investigated using a novel substrate trapping approach in which mutant or altered forms of the mammalian PTP, also referred to as substrate trapping PTPs, are used to bind (trap) one or more substrates of the PTP. Binding of the substrate trapping PTP with a substrate of the PTP results in the formation of a complex which can be readily observed, and, if desired, isolated, and characterized. The mutant forms of the PTPs have attenuated catalytic activity (lack catalytic activity or have reduced catalytic activity) relative to the wild type PTP but retain the ability to bind tyrosine phosphorylated substrate(s) of the wild type PTP.

The methods of the present invention are specifically exempliefied herein with respect to the phosphatases PTP1B and PTP-PEST; however, it is understood that the invention is not limited to these specific PTPs but is applicable to all members of the PTP family. In order to identify potential substrates of PTP1B and PTP-PEST, mutant (i.e., altered or substrate trapping) forms of PTP1B and PTP-PEST were generated which were catalytically attenuated but retained the ability to bind substrates. These mutant PTPs associated in stable complexes with proteins which were identified by immunoblotting as p210 bcr:abl and p130^{cas}, respectively. These associations were observed in lysates from several cell lines and in transfected COS cells, indicating that p210 bcr:abl and p130^{cas} represent major physiologically relevant substrates for PTP1B and PTP-PEST.

These results provide the first demonstration of PTPs having inherently restricted substrate specificity in vivo.

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The methods used to identify p210 bcr:abl and p130^{cas} as
specific substrates for PTP1B and PTP-PEST, respectively,
are generally applicable to any member of the PTP family, of
which approximately 500 members have currently been
5   reported, and can be used to determine the physiological
substrates of other members of the PTP family.

One embodiment of the invention relates to novel
mutant PTPs in which the invariant aspartate residue is
replaced with an amino acid which does not cause significant
10 alteration of the Km of the enzyme but which results in a
reduction in Kcat to less than 1 per minute (less than
1 min^{-1}). These PTPs retain the ability to form a complex
with, or bind, their tyrosine phosphorylated substrates, but
are catalytically attenuated. In one embodiment, the
15 invention relates to the phosphatase PTP1B in which the
invariant aspartate residue at position 181 is replaced with
alanine (D181A). In another embodiment the invention
relates to the phosphatase PTP-PEST in which the invariant
aspartate residue at position 199 is replaced with an
20 alanine (D199A). Another embodiment of the invention
relates to a PTP-PEST phosphatase in which the cysteine
residue at position 231 is replaced with a serine (C231S).
The invention also relates to other mutant or substrate
trapping PTPs in which the invariant aspartate residue is
25 replaced with or changed to another amino acid residue, such
as alanine. The invariant aspartate residue can be
identified in other PTPs by aligning the PTP nucleotide
sequence with the nucleotide sequence of a PTP for which the
location of the invariant aspartate residue is known.
30   The invention also relates to a method of identifying
a tyrosine phosphorylated substrate of a protein tyrosine
phosphatase. According to one embodiment of the present
invention, a tyrosine phosphorylated protein of interest is
combined with one or more PTP(s) in which the invariant
35 aspartate residue is replaced with an amino acid which does

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not cause significant alteration of the Km of the enzyme but
which results in a reduction in Kcat to less than 1 per
minute (less than 1 min⁻¹), and the presence or absence of a
complex between the protein and the PTP(s) is determined.
5 Presence of a complex in the combination indicates that the
tyrosine phosphorylated protein is a substrate of the PTP.
The PTP DA mutant binds to or complexes with its substrate
but does not dephosphorylate it (or does so very slowly),
thereby allowing the complex to be observed and, optionally,
10 isolated and identified. In a particular embodiment of the
invention, the invariant aspartate is replaced with an
alanine residue (a PTP DA mutation or alteration)

        In an alternative embodiment of the present invention,
a PTP of interest in which the invariant aspartate residue
15 is replaced with an amino acid which does not cause
significant alteration of the Km of the enzyme but which
results in a reduction in Kcat to less than 1 per minute
(less than 1 min⁻¹), is combined with one or more tyrosine
phosphorylated proteins, and the presence or absence of a
20 complex between the protein(s) and the PTP is determined.
Presence of a complex in the combination indicates that the
tyrosine phosphorylated protein is a substrate of the PTP.
The PTP DA mutant binds to or complexes with its substrate
but does not dephosphorylate it (or does so very slowly),
25 thereby allowing the complex to be observed, and,
optionally, isolated and identified. In one embodiment of
the invention, the invariant aspartate residue is replaced
with an alanine residue (a PTP DA mutation or alteration)

        The present invention also relates to a method of
30 identifying a tyrosine phosphorylated substrate of a protein
tyrosine phosphatase wherein more than one tyrosine
phosphorylated protein of interest is combined with more
than one PTP of interest in which the invariant aspartate
residue is replaced with an amino acid which does not cause
35 significant alteration of the Km of the enzyme but which

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results in a reduction in Kcat to less than 1 per minute
(less than 1 min^{-1}) (e.g., the invariant aspartate is
replaced with an alanine residue). Complexes formed in the
combination can be isolated and the component PTP and
5 substrate can be identified.

The invention also pertains to a method of reducing
the activity of a tyrosine phosphorylated protein,
comprising administering to a mammal a PTP in which the
invariant aspartate residue is replaced with an amino acid
10 which does not cause significant alteration of the Km of the
enzyme but which results in a reduction in Kcat to less than
1 per minute (less than 1 min^{-1}) (e.g., the invariant
aspartate is replaced with an alanine residue) and which
forms a complex with the tyrosine phosphorylated protein.
15 The PTP mutant binds to the phosphorylated protein without
dephosphorylating it, thereby inhibiting the activity of the
protein and reducing its downstream effects.

For example, the invention relates to a method of
reducing the transforming effects of oncogenes associated
20 with $p130^{cas}$, a substrate of PTP-PEST, comprising
administering to a mammal wild type PTP-PEST or PTP-PEST in
which the invariant aspartate residue is replaced with an
alanine residue. Wild type PTP-PEST binds and
dephosphorylates $p130^{cas}$, thereby negatively regulating its
25 downstream effects. DA mutants of PTP-PEST bind but do not
dephosphorylate $p130^{cas}$ (or dephosphorylate it at a reduced
rate); the substrate is thus tied up in the complex with the
substrate trapping form of PTP-PEST and cannot exert its
downstream effects. Similarly, the invention relates to a
30 method of reducing the formation of signalling complexes
associated with $p130^{cas}$, particularly those signalling
complexes which induce mitogenic pathways, comprising
administering to a mammal wild type PTP-PEST or PTP-PEST in
which the invariant aspartate residue is replaced with an
35 alanine residue.

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The present invention also relates to assays for identifying agents which alter, e.g., enhance or inhibit, the interaction between a PTP and its phosphorylated substrate. Agents identified by these assays can be
5   agonists (e.g., agents which enhance or increase the activity of the PTP) or antagonists (e.g., agents which inhibit or decrease the activity of the PTP) of PTP activity. The agent may be an endogenous physiological substance or may be a natural or synthetic drug, including
10   small organic molecules.

For example, the tyrosine phosphorylated substrate of a PTP can be identified by the methods described herein. An enzymatic activity assay utilizing the wild type PTP can be carried out in the presence of an agent to be tested, and
15   the resulting amount of enzyme activity can be compared with the amount of enzyme activity in the absence of the agent to be tested. A decrease in the enzymatic activity in the presence of the agent to be tested indicates that the agent inhibits the interaction between the PTP and its substrate.
20   Conversely, an increase in the enzymatic activity in the presence of the agent to be tested indicates that the agent enhances the interaction between the PTP and its substrate.

Alternatively, a competitive binding assay can be carried out utilizing the mutant PTP in the presence of an
25   agent to be tested, and the resulting extent of binding of the mutant PTP to its substrate can be compared with the extent of binding in the absence of the agent to be tested. A decrease in the extent of binding in the presence of the agent to be tested indicates that the agent inhibits the
30   interaction between the PTP and its substrate. Conversely, an increase in the extent of binding in the presence of the agent to be tested indicates that the agent enhances the interaction between the PTP and its substrate.

Thus, the compositions and methods described herein
35   are useful in identifying the tyrosine phosphorylated

substrates of members of the PTP family of phosphatases, as
well as in regulating the activity of identified substrates.
The compositions and methods described herein are also
useful for identifying tyrosine phosphorylated proteins
5   which are related to a particular disease or disorder, and
to methods of screening for modulators which enhance or
inhibit the PTP/substrate interaction for use in therapeutic
applications.

Brief Description of the Drawings

10   Figures 1A and 1B show a multiple sequence alignment
of the catalytic domains of PTPs. In Figure 1A, cytosolic
eukaryotic PTPs and domain 1 of RPTPs are combined into one
group, domains 2 of RPTPs are in a second group, and the
Yersinia PTP is in a third. Invariant residues shared among
15 all three groups are shown in red. Invariant and highly
conserved residues within a group are shown in blue and
green, respectively. Within the Yersinia PTP sequence,
residue that are either invariant or highly conserved
between the cytosolic and RPTP domain sequences are colored
20 blue and green, respectively. The position of residues of
PTP1B that interact with the peptide are indicated with a
red arrow, and the residue numbering at the bottom of the
alignment corresponds to that for PTP1B. Figure 1B is a
black and white photocopy of Figure 1A in which the colored
25 areas are indicated with labeled arrows.
        Figure 2 shows the Vmax and Km of various PTP1B
mutants toward RCML.

Detailed Description of the Invention
        The PTP family of enzymes contains a common
30 evolutionarily conserved segment of approximately 250 amino
acids known as the PTP catalytic domain. Within this

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conserved domain is a unique signature sequence motif,
[I/V]HCXAGXXR[S/T]G, that is invariant among all PTPs. The
cysteine residue in this motif is invariant in members of
the family and is known to be essential for catalysis. It
5 functions as a nucleophile to attack the phosphate moiety of
the incoming substrate. If the cysteine residue is altered
by site-directed mutagenesis to serine (CS mutants) or
alanine (CA mutants), the resulting PTP is catalytically
attenuated but retains the ability to complex with, or bind,
10 its substrate, at least in vitro. These results have been
confirmed relative to MKP-1, a member of the PTP family (Sun
et al., Cell 75:487-493 (1993)), as well as other PTPs.
However, although these CS mutants can in general bind
effectively to phosphotyrosyl substrates in vitro, in many
15 cases such complexes cannot be isolated in vivo. Thus, the
CS mutants are limited in their applicability and cannot be
used to isolate all combinations of PTPs and substrates.

The crystal structures of PTP1B alone (Barford, et
al., Science 263:1397-1404 (1994)) and in a complex with a
20 phosphotyrosine-containing peptide (Jia et al., Science
268:1754-1758 (1995)) were recently determined. These
structures indicated twenty seven invariant residues
(Barford et al., 1994), one of which is an aspartate
residue. This aspartate residue is invariant across the
25 catalytic domains of PTP family members. That is, if the
amino acid sequences of the PTP family members are aligned,
the aspartate residue is present in each PTP at a
corresponding location, although the position numbers may be
different due to the shifts required to maximize alignment
30 (see the Figure (from Barford et al., Nature Struc. Biol.
2:1043-1053 (1995)) for an alignment of various PTP
sequences). Sequences for which the alignment has not yet
been published can readily be aligned with other known PTP
sequences, e.g., utilizing available computer software such
35 as GENEWORKS.

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Thus, mutant PTPs other than those specifically
described herein can readily be made by aligning the amino
acid sequence of the PTP catalytic domain with those
described herein, identifying the invariant aspartate
5   residue, and changing the residue by site-directed
mutagenesis. Although the specific examples of PTP mutants
described herein are aspartate to alanine mutants (DA
mutants), it is understood that the invention is not limited
to changes of aspartate to alanine. The invariant aspartate
10   residue can be changed, e.g., by site-directed mutagenesis,
to any amino acid which does not cause significant
alteration of the Km of the enzyme but which results in a
reduction in Kcat to less than 1 per minute (less than
1 min⁻¹). For example, the invariant aspartate residue can
15   be changed or mutated to an alanine, valine, leucine,
isoleucine, proline, phenylalanine, tryptophan, methionine,
glycine, serine, threonine, cysteine, tyrosine, asparagine,
glutamine, lysine, arginine or histidine.

As described herein, pervanadate-treated cells were
20   used as an abundant source of tyrosine phosphorylated
proteins to investigate the substrate specificity of PTP-
PEST. PTP-PEST is an 88 kDa cytosolic PTP (Charest et al.,
Biochem. J. 308:425-432 (1995); den Hertog et al., Biochem.
Biophys. Res. Commun. 184:1241-1249 (1992); Takekawa et al.,
25   Biochem. Biophys. Res. Commun. 189:1223-1230 (1992); Yang et
al., J. Biol. Chem. 268:6622-6628 (1993); Yang et al., J.
Biol. Chem. 268:17650 (1993)) which is expressed
ubiquitously in mammalian tissues (Yi et al., Blood 78:
2222-2228 (1991)), and which exhibits high specific activity
30   when assayed in vitro using artificial tyrosine
phosphorylated substrates (Garton and Tonks, EMBO J.
13:3763-3771 (1994)). It has previously been demonstrated
that PTP-PEST is subject to regulation via phosphorylation
of Ser39 in vitro and in vivo. This modification is
35   catalyzed by both protein kinase C (PKC) and protein kinase

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A (PKA), and results in reduced enzyme activity as a consequence of an increase in the Km of the dephosphorylation reaction (Garton and Tonks, EMBO J. 13:3763-3771 (1994)). It appears likely that further regulatory mechanisms exist for PTP-PEST, since this enzyme would be expected to exert a considerable negative influence on the tyrosine phosphorylation state of cytosolic substrates of tyrosine kinases. One possibility is that this influence could be limited by the substrate specificity of PTP-PEST.

The substrate specificity of PTP1B was investigated utilizing the same methods outlined for PTP-PEST, with the exception that the cells were not treated with pervanadate. A combination of in vitro dephosphorylation and substrate trapping experiments were used to study the substrate interactions of PTP1B and PTP-PEST. The substrate trapping methods outlined herein are generally applicable to any PTP by virtue of the shared invariant aspartate residue, and should therefore prove useful in delineating the substrate preference of other PTP family members. In particular, the use of mutant, catalytically impaired PTPs to trap, and thereby isolate, potential substrates will greatly facilitate the identification of physiologically important substrates for individual PTPs, leading to improved understanding of the roles of these enzymes in regulation of cellular processes.

One embodiment of the invention relates to novel PTPs in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}). These PTPs retain the ability to form a complex with, or bind, their tyrosine phosphorylated substrates but are catalytically attenuated. As defined herein, "attenuated" activity is intended to mean that the phosphatase retains a

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similar Km to that of the wild type phosphatase but has a
Vmax which is reduced by a factor of at least 10^4 relative
to the wild type enzyme. This includes catalytic activity
which is either reduced or abolished relative to the wild

5   type PTP. For example, the invariant aspartate residue can
be changed or mutated to an alanine, valine, leucine,
isoleucine, proline, phenylalanine, tryptophan, methionine,
glycine, serine, threonine, cysteine, tyrosine, asparagine,
glutamine, lysine, arginine or histidine.

10   The novel PTPs described herein, in which the
invariant aspartate residue is replaced with an amino acid
which does not cause significant alteration of the Km of the
enzyme but which results in a reduction in Kcat to less than
1 per minute (less than 1 min^{-1}), can also comprise other

15   mutations, particularly those which assist in stabilizing
the PTP/substrate complex. For example, a mutation of the
[serine/threonine] residue in the signature motif to an
alanine residue changes the rate-determining step of the
dephosphorylation reaction from the formation of the

20   transition state to the break down of the transition state,
thereby stabilizing the PTP/substrate complex. Such
mutations may be valuably combined with the replacement of
the invariant aspartate residue, particularly assisting in
stabilizing the complex and facilitating the observation and

25   isolation of the complex.

        PTPs suitable for use in the invention include any PTP
which has an invariant aspartate residue in a corresponding
position. As defined herein, a phosphatase is a member of
the PTP family if it contains the signature motif

30   [I/V]HCXAGXXR[S/T]G. Dual specificity PTPs, i.e., PTPs
which dephosphorylate both phosphorylated tyrosine and
phosphorylated serine or threonine, are also suitable for
use in the invention. Appropriate PTPs include, but are not
limited to, PTP1B, PTP-PEST, PTP$_\gamma$, MKP-1, DEP-1, PTPμ,

35   PTPX1, PTPX10 and PTPH1.

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In one embodiment, the invention relates to the
phosphatase PTP1B in which the aspartate residue at position
181 is replaced with alanine (D181A). In another embodiment
the invention relates to the phosphatase PTP-PEST in which

5   the invariant aspartate residue at position 199 is replaced
with an alanine (D199A). Another embodiment of the
invention relates to a PTP-PEST phosphatase in which the
cysteine residue at position 231 is replaced with a serine
(C231S).

10   The invention also relates to a method of identifying
a tyrosine phosphorylated protein which is a substrate of a
particular protein tyrosine phosphatase. According to one
embodiment of the present invention, a tyrosine
phosphorylated protein of interest is combined with at least

15   one PTP in which the invariant aspartate residue is replaced
with an amino acid which does not cause significant
alteration of the Km of the enzyme but which results in a
reduction in Kcat to less than 1 per minute (less than
1 min^{-1}) (e.g., an alanine residue), and the presence or

20   absence of a complex between the protein and the PTP is
determined. Presence of a complex in the combination
indicates that the tyrosine phosphorylated protein is a
substrate of the PTP. The PTP DA mutant (substrate trapping
mutant) binds to or complexes with its substrate but does

25   not dephosphorylate it (or does so very slowly), thereby
allowing the complex to be isolated and identified.

The phosphorylated protein/PTP complex may be isolated
by conventional isolation techniques as described in U.S.
Patent No. 5,352,660 to Pawson, including salting out,

30   chromatography, electrophoresis, gel filtration,
fractionation, absorption, polyacrylamide gel
electrophoresis, agglutination, or combinations thereof.
Furthermore, to facilitate the determination of the presence
of the protein/PTP complex, antibodies against the PTP or

35   the phosphorylated protein can be used, as well as labelled

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PTPs and/or labelled phosphorylated substrates. The PTP or
phosphorylated protein can be labelled with various enzymes,
fluorescent materials, luminescent materials and radioactive
materials. Examples of suitable enzymes include, but are

5   not limited to, horseradish peroxidase, biotin, alkaline
phosphatase, β-galactosidase and acetylcholinesterase.
Examples of suitable fluorescent materials include, but are
not limited to, umbelliferone, fluorescein, fluorescein
isothiocyanate, rhodamine, dichlorotriazinylamine

10 fluorescein, dansyl chloride and phycoerythrin. Appropriate
luminescent materials include luminol, and suitable
radioactive material include radioactive phosphorous ^{32}P,
iodine I^{125}, I^{131} or tritium.

        Alternatively, the invention pertains to a method of

15 identifying a tyrosine phosphorylated protein which is a
substrate of a PTP, comprising combining a PTP of interest
in which the invariant aspartate residue is replaced with an
amino acid which does not cause significant alteration of
the Km of the enzyme but which results in a reduction in

20 Kcat to less than 1 per minute (less than 1 min^{-1}) (e.g., an
alanine residue), with at least one tyrosine phosphorylated
protein, thereby producing a combination; and determining
the presence or absence of a complex in the combination,
wherein presence of a complex in the combination between a

25 tyrosine phosphorylated protein and the PTP indicates that
the tyrosine phosphorylated protein is a substrate of the
PTP.

        The substrate trapping PTPs of the present invention
can also be used in place of wild type PTPs to screen

30 phosphotyrosyl peptide libraries for peptides which bind to
the PTP as described in Songyang et al. (Nature 373:536-539
(1995); Cell 72:767-778 (1993)). Peptides identified from
such peptide libraries can then be assessed to determine
whether tyrosine phosphorylated proteins containing these

35 peptides exist in nature.

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Any tyrosine phosphorylated protein is suitable as a potential substrate in the present invention. Tyrosine phosphorylated proteins are well known in the art. Specific examples of appropriate substrates include, without
5   limitation, p130^{cas}, the EGF receptor, p210 bcr:abl, MAP kinase and the insulin receptor. Of particular interest are tyrosine phosphorylated proteins which have been implicated in a mammalian disease or disorder.

The invention also pertains to a method of reducing
10   the activity of a tyrosine phosphorylated protein, comprising administering to a mammal a PTP in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than
15   1 per minute (less than 1 min⁻¹) (e.g., an alanine residue) and which forms a complex with the tyrosine phosphorylated protein. The PTP DA mutant binds to the phosphorylated protein without dephosphorylating it (or causing dephsophorylation at a greatly reduced rate), thereby
20   inhibiting the activity of the protein and reducing its downstream effects. As used herein, "reducing" includes both reduction and complete abolishment, e.g., of one or more activities or functions of the phosphorylated protein.

For example, the invention relates to a method of
25   reducing the transforming effects of oncogenes associated with p130^{cas}, a substrate of PTP-PEST, comprising administering to a mammal wild type PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an alanine residue. Wild type PTP-PEST binds and
30   dephosphorylates p130^{cas}, thereby negatively regulating its downstream effects. DA mutants of PTP-PEST bind but do not dephosphorylate p130^{cas} (or do so at a greatly reduced rate); the substrate is thus tied up in the complex with the substrate trapping form of PTP-PEST and cannot exert its
35   downstream effects. Similarly, the invention relates to a

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method of reducing the formation of signalling complexes associated with p130^{cas}, particularly those signalling complexes which induce mitogenic pathways, comprising administering to a mammal wild type PTP-PEST or PTP-PEST in

5   which the invariant aspartate residue is replaced with an alanine residue. The PTP binds to and/or dephosphorylates p130^{cas}, thereby negatively regulating the downstream effects of p130^{cas} and reducing the formation of signalling complexes associated with p130^{cas}.

10   The substrate trapping mutant PTPs of the present invention can be used in virtually any application in place of, or in addition to, a corresponding wild type PTP. The advantages of such a utility lie in the ability of the mutant PTP to mimic the function of the wild type enzyme,

15   e.g., to decrease the activity of its tyrosine phosphorylated substrate, without inducing the harmful cytotoxic effects commonly observed with administration or overexpression of the wild type PTP. Thus, the invention also pertains to a method of reducing the cytotoxic effects

20   associated with administration or overexpression of wild type PTPs. For example, CS mutants of MKP-1 have been shown to have the same functional effect as wild type MKP-1 without induction of potentially harmful side effects. Thus, PTPs described herein, in which the invariant

25   aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}) (e.g., an alanine residue), can be used in many applications in place of the corresponding

30   wild type enzyme. As used herein, a "corresponding" enzyme is one which is the same as the mutant PTP (e.g., PTP-PEST and PTP-PEST D199A) or one which is different from the mutant PTP but recognizes the same substrate as the mutant PTP.

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The mutant PTPs described herein can also be used therapeutically to reduce the activity of a tyrosine phosphorylated protein, such as by a gene therapy method in which the mutant PTP described herein, or a functional
5   portion thereof which retains the ability to bind to its tyrosine phosphorylated substrate, is introduced into a subject and in whom the mutant PTP is expressed. The mutant PTP replaces, either partially or totally, the wild type enzyme which is normally produced or competes with the wild
10 type PTP for binding to the substrate. For example, a specific tyrosine phosphorylated protein can be identified which is implicated in a particular disease or disorder (such as a protein tyrosine kinase). At least one PTP which acts to dephosphorylate the selected tyrosine phosphorylated
15 protein of the present invention can be identified by the methods described herein. The wild type or mutant form of the PTP can be administered to a subject in need of treatment in order to tie up or bind the tyrosine phosphorylated substrate, thereby inhibiting or reducing the
20 function of the phosphorylated protein. In a preferred embodiment, the mutant PTP is administered in place of the wild type enzyme in order to reduce the cytotoxic effects associated with overexpression of the wild type enzyme. Procedures for gene therapy are known in the art (see U.S.
25 Patent No. 5,399,346 to Anderson et al.) and can be modified by methods known in the art to appropriately express the specific mutant and wild type PTPs of the present invention.

The present invention also relates to assays for identifying agents which alter, e.g., enhance or inhibit,
30 the interaction between a PTP and its phosphorylated substrate. Agents identified by these assays can be agonists (e.g., agents which enhance or increase the activity of the PTP) or antagonists (e.g., agents which inhibit or decrease the activity of the PTP) of PTP
35 activity. The agent may be an endogenous physiological

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substance or may be a natural or synthetic drug, including small organic molecules.

For example, the tyrosine phosphorylated substrate of a PTP can be identified by the methods described herein. An
5   enzymatic activity assay utilizing the wild type PTP can be carried out in the presence of an agent to be tested, and the resulting amount of enzyme activity can be compared with the amount of enzyme activity in the absence of the agent to be tested. Enzymatic activity assays are known in the art;
10 for example, assays of PTP activity using a tyrosine phosphorylated ^{32}P-labelled substrate are described in Flint et al. (EMBO J. 12:1937-1946 (1993)). A decrease in the enzymatic activity in the presence of the agent to be tested indicates that the agent inhibits the interaction
15 between the PTP and its substrate. Conversely, an increase in the enzymatic activity in the presence of the agent to be tested indicates that the agent enhances the interaction between the PTP and its substrate.

Alternatively, a competitive binding assay can be
20 carried out utilizing the mutant PTP in the presence of an agent to be tested, and the resulting extent of binding of the mutant PTP to its substrate can be compared with the extent of binding in the absence of the agent to be tested. Competitive binding assays are known in the art; for
25 example, U.S. Patent No. 5,352,660 to Pawson describes methods suitable for use in this invention. A decrease in the extent of binding in the presence of the agent to be tested indicates that the agent inhibits the interaction between the PTP and its substrate. Conversely, an increase
30 in the extent of binding in the presence of the agent to be tested indicates that the agent enhances the interaction between the PTP and its substrate.

According to the present invention, tyrosine phosphorylated peptides identified with mutant PTPs from
35 peptide libraries by the methods of Songyang et al. (Nature

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<u>373</u>:536-539 (1995); <u>Cell</u> <u>72</u>:767-778 (1993)) can be used
herein in place of the complete tyrosine phosphorylated
protein in competitive binding assays.

The present invention also pertains to pharmaceutical
5   compositions comprising a PTP in which the invariant
aspartate residue is replaced with an amino acid which does
not cause significant alteration of the Km of the enzyme but
which results in a reduction in Kcat to less than 1 per
minute (less than 1 min^{-1}) (e.g., an alanine residue). For
10 instance, the PTP of the present invention can be formulated
with a physiologically acceptable medium to prepare a
pharmaceutical composition. The particular physiological
medium may include, but is not limited to, water, buffered
saline, polyols (e.g., glycerol, propylene glycol, liquid
15 polyethylene glycol) and dextrose solutions. The optimum
concentration of the active ingredient(s) in the chosen
medium can be determined empirically, according to
procedures well known to medicinal chemists, and will depend
on the ultimate pharmaceutical formulation desired. Methods
20 of introduction of exogenous PTPs at the site of treatment
include, but are not limited to, intradermal, intramuscular,
intraperitoneal, intravenous, subcutaneous, oral and
intranasal. Other suitable methods of introduction can also
include rechargeable or biodegradable devices and slow
25 release polymeric devices. The pharmaceutical compositions
of this invention can also be administered as part of a
combinatorial therapy with other agents.

The following Examples are offered for the purpose of
illustrating the present invention and are not to be
30 construed to limit the scope of this invention. The
teachings of all references cited herein are hereby
incorporated by reference in their entirety.

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EXAMPLES

Materials and Methods

The following is a description of the materials and methods used in the work described herein.

5 ### Generation, Expression and Purification of Mutant PTP Proteins

Point mutations within the catalytic domains of PTP-PEST (D199A, C231S) and PTP1B (D181A, C215S) were introduced by site-directed mutagenesis using the Muta-Gene^{TM} _in vitro_

10 mutagenesis kit (Bio-Rad, Richmond, CA). Regions containing the required point mutation were then exchanged with the wild type sequences within appropriate expression vectors, and the replaced mutant regions were sequenced in their entirety to verify the absence of additional mutations.

15 Full length PTP-PEST proteins (wild type and mutant proteins, containing either Asp199 to Ala or Cys231 to Ser mutations) and the wild type PTP-PEST catalytic domain (amino acids 1-305) were expressed in Sf9 cells using recombinant baculovirus (BaculoGold^{TM}, Pharmingen, San

20 Diego, CA), and purified as described in Garton and Tonks (_EMBO J._ 13:3763-3771 (1994)). Truncated forms of wild type and mutant PTP-PEST proteins, comprising amino acid residues 1-305 of PTP-PEST were also expressed in _E. coli_ as GST fusion proteins following subcloning of PTP-PEST DNA in-

25 frame downstream of GST in pGEX vectors (Pharmacia Biotech Inc., Uppsala, Sweden). Twenty-five ml of _E. coli_ transformed with the appropriate vector were grown to log phase (OD$_{600}$ approximately 0.5). Fusion protein expression was then induced by addition of 0.2 mM isopropyl-1-thio-b-D-

30 galactopyranoside, and the cells were grown for 2-4 hours at 30°C. Cells were harvested by centrifugation, incubated with 50 mg/ml lysozyme in 3 ml buffer containing 50 mM Tris-HCl, pH 7.4, 5mM EDTA, 1 mM PMSF, 1 mM benzamidine, 5 mg/ml leupeptin, 5 mg/ml aprotinin, 0.1 % Triton X-100 and 150 mM

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NaCl, then lysed by sonication (3 x 10s). Following removal of insoluble material by centrifugation (20 minutes at 300,000 x g), fusion proteins were isolated by incubation for 30 min at 4°C with 100 ml glutathione-Sepharose beads (Pharmacia Biotech Inc., Uppsala, Sweden), and the beads were then collected by centrifugation and washed three times with buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 10 % glycerol, 1 % Triton X-100 and 100 mM NaCl). This procedure yielded essentially homogeneous fusion protein at a concentration of 1 mg protein/ml glutathione-Sepharose beads. PTP1B proteins (wild type and mutant forms) comprising amino acids 1-321 were expressed in E.coli and purified to homogeneity as described in Barford et al. (J. Mol. Biol. 239:726-730 (1994)).

Cell Culture, Transfection, Preparation of Lysates and Fractionation

HeLa and COS cells were grown in Dulbecco's modified Eagle's medium (DMEM), containing 5% fetal bovine serum (FBS); Wi38, C2C12 and MvLu cells were grown in DMEM containing 10% FBS; 293 cells were grown in DMEM containing 10% calf serum; MCF10A cells were grown in 50% DMEM, 50% Ham's F-12 containing 5% horse serum, 20 ng/ml epidermal growth factor, 10 mg/ml insulin, 0.5 mg/ml hydrocortisone and 0.25 mg/ml fungizone. All media also contained penicillin and streptomycin at 100 U/ml and 100 mg/ml, respectively, and all cells were grown at 37°C. Calcium phosphate-mediated transfection was used to introduce cDNA encoding wild type and mutant PTP-PEST proteins into COS cells. These were encoded by PTP-PEST cDNA subcloned into the plasmid PMT2, from which expression was driven by an adenovirus major late promoter; 20 mg DNA was used for transfection of each 10 cm plate of cells. The level of expression of PTP-PEST constructs was similar in all cases.

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Prior to cell lysis, 70-90% confluent cultures of cells were treated for 30 minutes with 0.1 mM pervanadate (20 ml of a fresh solution containing 50 mM sodium metavanadate ($NaVO_3$) and 50 mM H_2O_2 were added to 10 ml

5   medium). Treatment of cells with H_2O_2 and vanadate leads to a synergistic increase in phosphotyrosine levels, presumably due to inhibition of intracellular PTPs by vanadate. The synergism between H_2O_2 and vanadate has previously been suggested to result from improved

10   accumulation of the resultant oxidized vanadate (pervanadate) within the cells when compared to vanadate itself (Heffetz et al., J. Biol. Chem. 265:2896-2902 (1990)). It is important to note that during the preparation of cell lysates, dilution occurs such that the

15   inhibitory effect of vanadate on PTP action is lost. Pervanadate treatment resulted in the appearance of at least 50 prominent phosphotyrosine protein bands in all cell types, whereas untreated cells contained virtually undetectable levels of phosphotyrosine (data not shown).

20   Cells were lysed in Buffer A containing 5 mM iodoacetic acid, which was included in order to inhibit irreversibly cellular PTPs. Following incubation at 4°C for 30 minutes, 10 mM DTT was added to inactivate any unreacted iodoacetic acid. Insoluble material was then removed by

25   centrifugation for 20 minutes at 300,000 x g. The resultant lysates were stable with regard to their phosphotyrosine content during long term (several months) storage at -70°C and during prolonged (at least 20 hours) incubation at 4°C, in the absence of exogenous added PTPs.

30   Pervanadate-treated HeLa cell lysate was fractionated by anion exchange chromatography using a Mono Q FPLC column (Pharmacia). The sample (50 mg total protein at 3 mg/ml in buffer A) was diluted in three volumes of buffer B (20 mM tris-HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine, 1 mg/ml

35   leupeptin, 1 mg/ml aprotinin and 0.1% Triton X-100) prior to

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loading. Proteins were eluted at a flow rate of 1 ml/min
with a linear gradient of 0-0.5 M NaCl in buffer B over 20
fractions (1 ml fraction volume), followed by a second
gradient of 0.5-1.0 M NaCl in buffer B over 5 fractions.

5 Phosphotyrosine-containing proteins were detected within
fractions 7-21 according to anti-phosphotyrosine
immunoblotting. The same procedures were followed for
PTP1B, with the exception that the cells were not treated
with pervanadate.

10 Dephosphorylation Reactions

        Lysates of pervanadate-treated HeLa cells (1-2 mg
protein/ml) containing tyrosine phosphorylated proteins were
incubated on ice in the absence or presence of purified
active PTPs at a concentration of 1 nM. Dephosphorylation

15 was terminated by the removal of aliquots (30 mg protein)
into SDS-PAGE sample buffer, and the extent of
dephosphorylation was determined by immunoblotting using the
monoclonal antibody G104. Assays of PTP activity using
tyrosine phosphorylated ^{32}P-labelled RCM-lysozyme as

20 substrate were performed as described in Flint et al. (EMBO
J. 12:1937-1946 (1993)).

    Antibodies and Immunoblotting

        The PTP-PEST monoclonal antibody AG25 was raised
against baculovirus-expressed purified full-length PTP-PEST.

25 The anti-phosphotyrosine monoclonal antibody G104 was
generated using as antigen phosphotyrosine, alanine and
glycine, in a 1:1:1 ratio, polymerized in the presence of
keyhole limpet hemocyanin with 1-ethyl-3-(3'-
dimethylaminopropyl)carbodiimide, a method originally

30 described in Kamps and Sefton (Oncogene 2:305-315 (1988)).
p130^{cas} monoclonal antibody was from Transduction
Laboratories (Lexington, Ky). Monoclonal antibody FG6
against PTP1B was provided by Dr David Hill (Calbiochem

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Oncogene Research Products, Cambridge, MA). Visualization
of proteins by immunoblotting was achieved by enhanced
chemiluminescence (ECL) using HRP-conjugated secondary
antibodies (Amersham Life Science Inc., Arlington Heights,
5   Il) and the SuperSignal^{TM} CL-HRP substrate system (Pierce,
Rockford, Il).

Immunoprecipitation and Substrate Trapping

Immunoprecipitation of PTP-PEST from transfected COS
cells was performed following covalent coupling of
10 monoclonal antibody AG25 to protein A-Sepharose beads
(Pharmacia Biotech Inc., Uppsala, Sweden) using the chemical
cross-linking agent dimethyl pimelimidate (Schneider et al.,
J. Biol. Chem. 257:10766-10769 (1982)). Antibody was first
bound to protein A-Sepharose at a concentration of 1 mg/ml
15 bead volume, and unbound material was then removed by three
washes with 0.2 M sodium borate, pH 9. Covalent coupling
was achieved by incubation at room temperature for 30
minutes in the presence of 20 mM dimethyl pimelimidate in
0.2 M sodium borate, pH 9. The beads were then incubated
20 for 1 hour with an excess of 0.2 M ethanolamine, pH 8, to
block any unreacted cross-linker, and washed three times
with PBS prior to storage at 4°C. Ten ml of AG25 beads were
used to precipitate transfected PTP-PEST from lysates
containing approximately 0.375 mg protein.
25   Substrate trapping was performed using various PTP
affinity matrices. The full-length PTP-PEST matrix utilized
covalent coupled AG25-protein A-Sepharose beads to which
purified baculovirus-expressed PTP-PEST protein was bound.
Aliquots (10ml) of AG25 beads were incubated for 2 hours at
30 4°C in 100 ml buffer A in the presence of 5 mg of purified
PTP-PEST (wild type or mutant forms); unbound PTP-PEST was
then removed by washing three times with 1 ml buffer A. The
resultant PTP-PEST-AG25-protein A-Sepharose beads contained
approximately 2 mg of PTP-PEST per 10 ml aliquot. Substrate

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trapping was also carried out with glutathione-Sepharose
beads bound to bacterially-expressed GST fusion proteins
containing the catalytic domain of PTP-PEST.

PTP1B was also used in substrate trapping experiments.
5   In this case, the monoclonal antibody FG6 was precoupled to
protein A-Sepharose in the absence of cross-linker (2 mg
antibody/10 ml beads), then purified PTP1B proteins were
added in excess and incubated at 4°C for 2 hours. Following
removal of unbound PTP1B, 10 ml beads contained
10   approximately 2 mg PTP1B.

Pervanadate-treated cell lysates, or column fractions,
were used as a source of phosphotyrosine-containing proteins
for substrate trapping experiments. In general, lysates
containing 0.25-0.5 mg protein in 0.5 ml buffer A (including
15   5 mM iodoacetic acid, 10 mM DTT) were incubated at 4°C for 2
hours in the presence of 10 ml of affinity matrix containing
approximately 2 mg of the appropriate PTP protein. Unbound
proteins were then removed from the samples by washing three
times with 1 ml buffer A, and bound material was collected
20   by addition of 50 ml SDS-PAGE sample buffer followed by
heating at 95°C for 5 minutes; proteins bound to the beads
were then analyzed by SDS-PAGE followed by immunoblotting.

Results

The following details the results of the work
25   described herein carried out as described above.

PTP1B and p210 bcr:abl

Chronic myelogenous leukemia (CML) is a clonal
disorder of the haematopoietic stem cell that is
characterized by the Philadelphia chromosome, in which the
30   c-Abl proto-oncogene on chromosome 9, encoding a PTK,
becomes linked to the bcr gene on chromosome 22. This
results in the generation of a bcr:able fusion protein, p210
bcr:abl, in which the PTK activity is enhanced relative to

that of c-Abl. Current data indicates that this cytogenetic
abnormality is the primary and sole causative event in CML.
Expression of p210 bcr:abl produces abnormal patterns of
tyrosine phosphorylation that result in the aberrant
5 maturation of the haematopoietic stem cell that is
characteristic of CML.

       Expression of PTP1B mRNA and protein is enhanced as a
consequence of p210 bcr:abl expression in Rat1, Mo7 and BaF3
cells. Changes in PTP1B activity, which were commensurate
10 with the change in enzyme protein, were also observed.
These changes are specific for PTP1B and are not seen in
closely related homologue (65% identity) TC-PTP or in other
tested PTPs, including SHP-1, SHP-2 and PTP-PEST. The
increase in expression of PTP1B was also observed in Ph+ B-
15 lymphoid cells derived from a CML patient relative to Ph-
cells from the same patient.

       The changes in PTP1B levels were induced specifically
by p210 bcr:abl and were not seen in cells expressing other
PTKs including v-abl, v-src or other oncoproteins such as
20 myc. The PTK activity of p210 bcr:abl was essential for the
increase in expression of PTP1B, since expression of an
inactive lysine to arginine mutant form of p210 bcr:abl in
Rat1 cells did not alter PTP1B levels. The increase in
PTP1B levels is a rapid response to induction of p210
25 bcr:abl. When BaF3 cells expressing a temperature-sensitive
mutant form of p210 bcr:abl were shifted to the permissive
temperature for the PTK, PTP1B levels were observed to
increase within 12-24 hours coincident with the appearance
of the active form of the PTK. THese data indicate that the
30 alteration PTP1B levels is a relatively rapid response to
the appearance of p210 bcr:abl, rather than a long-term
adaptive response of the cells.

       In transient cotransfection experiments in COS cells,
PTP1B dephosphorylates p210 bcr:abl but not v-abl. When the
35 PTP1B D181A mutant was expressed as a GST fusion protein,

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purified and incubated with lysates of Mo7-p210 cells (which overexpress p210 bcr:abl), a complex of the mutant PTP and p210 bcr:abl was isolated. In contrast, tyrosine phosphorylated c-abl, which was also present in the lysates,
5   did not bind to the mutant PTP. The interaction between PTP1B D181A and p210 bcr:abl was blocked by vanadate, suggesting that the interaction involved the active site of the PTP.

Following transient coexpression in COS cells, PTP1B
10   D181A formed a complex with p210 bcr:abl. Preliminary data indicate that the Y177F mutant form of p210 bcr:abl did not interact with PTP1b D181A, suggesting that this tyrosine residue is a component of the binding site in the PTK. This tyrosine residue in p210 bcr:abl is phosphorylated in vivo
15   and has been demonstrated to serve as a docking site for GRB2. Direct interaction of the pTyr in p210 bcr:abl and the SH2 domain of GRB2 is essential for the transforming activity of the PTK. Interaction of PTP1B D181A with p210 bcr:abl interferes with the association of the PTK with
20   GRB2. Taken together, these data suggest that p210 bcr:abl is a physiological substrate of PTP1B and that PTP1B may function as an antagonist of the oncoprotein PTK in vivo. The Vmax, Km and Kcat of 37 kDa PTP1B mutants toward RCML are shown in Figure 2.

25   PTP1B and the EGF Receptor
Expression of PTP1B D181A in COS cells leads to enhanced phosphorylation of tyrosyl residues in a 180 kDa protein and in proteins of 120 and 70 kDa. When a GST-PTP1B D181A fusion protein is expressed in COS cells and
30   precipitated on Glutathione-Sepharose, the 180 kDa, and smaller quantities of p120 and p70, were coprecipitated. The p180 protein was identified as the epidermal growth factor (EGF) receptor by immunoblotting. The identity of

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the p120 and p70 proteins is unclear; however, the latter is
not src, p62 or paxillin.

Expression of PTP1B D181A in COS cells induces
tyrosine phosphorylation of the EGF receptor in the absence
5   of its ligand, EGF, indicating that the mutant PTP is
exerting its effects in the intact cell and not post-lysis.
The equivalent D199A PTP-PEST mutant does not interact with
the EGF receptor, indicating the specificity of this
substrate interaction.

10 Autophosphorylation of the EGF receptor is required
for the interaction with PTP1B D181A. Mutants of the
receptor that are either kinase-dead or in which the
autophosphorylation sites have been deleted do not interact
with PTP1B D181A. In v-src-expressing cells, a plethora of
15 tyrosine phosphorylated proteins were observed, but
phosphorylation of the EGF receptor was not detected. Under
these conditions, PTP1B D181A bound predominantly to a 70
kDa tyrosine phosphorylated protein.

As a result of this work, it appears that PTP1B can
20 modulate EGF-induced signalling pathways, perhaps including
the pathways of many diseases, including breast cancer.

Preferential Dephosphorylation of a 130 kDa Phosphotyrosine-
Containing Protein by PTP-PEST

In order to investigate the substrate specificity of
25 PTP-PEST in vitro, aliquots of pervanadate-treated HeLa cell
lysates were incubated on ice, yielding 50-100 distinct
phosphotyrosine-containing proteins as judged by
immunoblotting of the cell lysate using the monoclonal anti-
phosphotyrosine antibody G104. Purified full-length PTP-
30 PEST (expressed in Sf9 cells using recombinant baculovirus),
PTP-PEST catalytic domain, or PTP1B catalytic domain (37 kDa
form) was then added to the lysate, and aliquots were
removed at various time points for analysis by SDS-PAGE
followed by anti-phosphotyrosine immunoblotting.

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Surprisingly, a prominent 130 kDa phosphotyrosine band (p130) was selectively dephosphorylated by PTP-PEST within 10 minutes, whereas the intensity of all the other bands was essentially unchanged even after 60 minutes of incubation
5 with PTP-PEST. Long incubations with higher concentrations of PTP-PEST (greater than 100-fold) resulted in the complete removal of all phosphotyrosine bands from the lysate. However, under all conditions tested, p130 was found to be dephosphorylated more rapidly than all other bands present.
10   The selective dephosphorylation of p130 by PTP PEST was also observed using a truncated form of the phosphatase (amino acid residues 1-305) which essentially contains only the catalytic domain of the enzyme. This result suggests that the striking substrate preference displayed by PTP PEST
15 in this analysis is an inherent property of the phosphatase catalytic domain, whereas the C-terminal 500 amino acid residues have little discernible effect on the substrate specificity of the enzyme.

The specificity of the interaction between PTP-PEST
20 and p130 was addressed using the catalytic domain of PTP1B (amino acid residues 1-321) in dephosphorylation reactions. When added at a similar molar concentration to that used for PTP-PEST, PTP1B was found to dephosphorylate fully and rapidly (within 15 minutes) most of the phosphotyrosine-
25 containing proteins present in the pervanadate-treated HeLa lysate. In addition, the time course of dephosphorylation of p130 was not significantly more rapid than that of the other phosphotyrosine bands dephosphorylated by PTP1B. It should be noted, however, that these in vitro
30 dephosphorylation results are not truly illustrative of the substrate specificity of PTP1B in vivo for several reasons. First, only the isolated catalytic subunit was used in this particular experiment. Furthermore, in vivo substrate specificity may be quite different due to the intracellular
35 distribution of both the PTP and potential substrates. That

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is, in vitro dephosphorylation experiemnts may utilize substrates which the PTP is capable of dephosphorylating but which it would not have access to in vivo. The phenomenon of differing substrate specificity depending upon different

5   physiologic contexts is illustrated by a comparison of this data with the in vivo PTP1B work described above, wherein PTP1B showed specificity for only three proteins.

Identification of Phosphotyrosine-Containing p130 Protein as p130^{cas} by Substrate Trapping

10 Pervanadate-treated HeLa cell lysate was fractionated by anion exchange chromatography and aliquots of the fractions were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine or anti-p130^{cas} antibodies. Aliquots of all samples analyzed were then

15 incubated with an affinity matrix containing a substrate trapping PTP-PEST mutant, comprising full length PTP-PEST in which Asp199 is changed to alanine (D199A), bound to covalently coupled protein A-Sepharose/antibody (AG25) beads. Proteins associated with PTP-PEST were then analyzed

20 by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine or anti-p130^{cas} antibodies.

Anti-phosphotyrosine immunoblotting of the column fractions showed that the p130 phosphotyrosine band eluted as a single peak in fractions 11-14 (approx. 0.3 M NaCl).

25 In view of the abundance of tyrosine phosphorylated p130 in HeLa lysates, it appeared likely that p130 represents a previously identified phosphotyrosine-containing 130 kDa protein. Several potential candidates were identified in the literature, including the focal adhesion kinase

30 p125^{FAK}, ras-GAP, gp130 and p130^{cas}. Of these candidates, p130^{cas} has been identified as a particularly prominent phosphotyrosine band in a wide variety of systems, including v-crk (Mayer and Hanafusa, Proc. Natl. Acad. Sci. USA 87: 2638-2642 (1990); Mayer et al., Nature 332:272-275 (1988))

and src (Kanner et al., Proc. Natl. Acad. Sci. USA 87:3328-
3332 (1990); Reynolds et al., Mol. Cell. Biol. 9: 3951-3958
(1989)) transformed fibroblasts, integrin mediated cell
adhesion (Nojima et al., J. Biol. Chem. 270:15398-15402

5   (1995); Petch et al., J. Cell Science 108:1371-1379 (1995);
Vuori and Ruoslahti, J. Biol. Chem. 270:22259-22262 (1995))
and PDGF stimulated 3T3 cells (Rankin and Rozengurt, J.
Biol. Chem. 269:704-710 (1994)).

     Therefore, the possibility that the p130

10 phosphotyrosine band corresponds to p130^{cas} was tested by
immunoblotting the Mono Q fractions using an antibody to
p130^{cas}. The 130 kDa band corresponding to p130^{cas} eluted
in the same fractions as the p130 tyrosine phosphorylated
band, and displayed a similar apparent molecular weight,

15 suggesting that they might represent the same protein.
Furthermore, p130^{cas} immunoprecipitated from these
fractions was found to be phosphorylated on tyrosyl
residues.

     A mutant form of PTP-PEST (D199A) was generated by

20 site-directed mutagenesis, and the mutant enzyme was
purified following expression using recombinant baculovirus.
When assayed using tyrosine phosphorylated RCM-Lysozyme as
substrate, the purified mutant enzyme exhibited a specific
activity which was approximately 10,000 fold lower than

25 that of the wild type enzyme (Garton and Tonks, unpublished
data). This purified protein was bound to an affinity
matrix comprised of an anti-PTP-PEST monoclonal antibody
(AG25) covalently coupled to Protein A-Sepharose beads, then
incubated with each of the Mono Q fractions. After 45

30 minutes of incubation, proteins associating with the mutant
PTP-PEST were collected by centrifugation, the beads were
washed, and SDS-PAGE sample buffer was added. Associated
proteins were then analyzed by immunoblotting using the
monoclonal anti-phosphotyrosine antibody G104.

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The mutant PTP-PEST protein was found to associate with a single phosphotyrosine-containing protein, the molecular weight (130 kDa) and Mono Q elution position (fractions 11-14) of which coincided with those of p130^{cas}.

5   Immunoblotting of the PTP-PEST-associated proteins using the p130^{cas} antibody demonstrated that the 130 kDa tyrosine phosphorylated protein trapped by the mutant PTP-PEST is indeed p130^{cas}. These data further support the hypothesis that p130^{cas} is a potential physiologically relevant

10   substrate for PTP-PEST.

Determination of Structural Features of PTP-PEST Involved in Specific Interaction with Tyrosine Phosphorylated p130^{cas}

The interaction between p130^{cas} and PTP-PEST was investigated further in substrate trapping experiments using

15   various purified mutant forms of PTP-PEST to precipitate proteins from pervanadate-treated HeLa lysates. Several affinity matrices were incubated with pervanadate-treated HeLa cell lysate, and proteins associated with the beads were analyzed by SDS-PAGE followed by immunoblotting with

20   anti-phosphotyrosine or anti-p130^{cas} antibodies.

The wild type full-length phosphatase was found to be incapable of stable association with tyrosine phosphorylated p130^{cas}, whereas both the PTP-PEST (D199A) mutant protein and a mutant lacking the active site cysteine residue

25   (C231S) specifically precipitated p130^{cas} from the lysate. The inability of the wild type phosphatase to precipitate tyrosine phosphorylated p130^{cas} presumably reflects the transient nature of the normal interaction between PTP-PEST and tyrosine phosphorylated p130^{cas}, which is likely to be

30   concluded as soon as p130^{cas} is dephosphorylated by PTP-PEST.

Since the C-terminal 500 amino acids of PTP-PEST contain several proline-rich regions which resemble src homology-3 (SH3) domain binding sequences, it appeared

plausible that the specificity of the interaction between
PTP-PEST and p130^{cas} might depend to some extent on
association of these segments with the SH3 domain of
p130^{cas}. The possible contribution of the C-terminal
5   segment of PTP-PEST in the observed specific interaction of
PTP-PEST with p130^{cas} was therefore addressed in further
substrate trapping experiments using GST fusion proteins
containing the catalytic domain of PTP-PEST alone, in both
wild type and mutant (D199A) forms. The mutant catalytic
10   domain of PTP-PEST fused to GST was found to precipitate the
p130^{cas} phosphotyrosine band specifically, whereas both the
wild type fusion protein and GST alone failed to precipitate
p130^{cas}. The specific interaction between PTP-PEST and
p130^{cas} observed in these experiments therefore appears to
15   be an intrinsic property of the catalytic domain of PTP-
PEST, emulating the observed preference of the active PTP-
PEST catalytic domain for dephosphorylation of p130^{cas} in
vitro.

Specificity of Interaction Between Mutant PTP-PEST and
20   Tyrosine Phosphorylated p130^{cas}

In view of the relative abundance of tyrosine
phosphorylated p130^{cas} in the pervanadate-treated HeLa cell
lysate, the possibility that the observed selective binding
of PTP-PEST inactive mutant proteins to p130^{cas} was
25   substrate-directed (reflecting the abundance of this
potential substrate relative to the other phosphotyrosine-
containing proteins present in the lysate) rather than
enzyme-directed (reflecting a genuine substrate preference
of PTP-PEST) was considered; this possibility was addressed
30   in two ways. First, inactive mutant forms of the catalytic
domain of PTP1B were used to trap potential substrates for
this enzyme from the pervanadate-treated HeLa lysates.
Again it was found that the wild type phosphatase was
incapable of stable interaction with any phosphotyrosine-

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containing protein, whereas mutant variants of the PTP1B
phosphatase domain (comprising Cys or Asp mutations
analogous to those described above for PTP-PEST) associated
with many tyrosine phosphorylated proteins. This was
5   especially apparent for the aspartic acid mutant of PTP1B
(D181A), which appeared to precipitate essentially all
phosphotyrosine-containing proteins from the lysate with
similar efficacy. These data emphasize the specific nature
of the interaction between PTP-PEST and p130^{cas}, which
10   appears to be a property peculiar to the PTP-PEST catalytic
domain, rather than a feature shared by all PTP catalytic
domains.

     The specificity of the interaction between PTP-PEST
and p130^{cas} was addressed further following pervanadate-
15   treatment of several different cell lines (Wi38, 293, COS,
MCF10A, C2C12, MvLu), yielding a different array of tyrosine
phosphorylated proteins in each case; the resultant lysates
were analyzed by SDS-PAGE followed by anti-phosphotyrosine
immunoblotting. Aliquots were incubated with PTP-PEST
20   (D199A) affinity matrix or control matrix, and tyrosine
phosphorylated proteins associating with PTP-PEST were
analyzed by SDS-PAGE and immunoblotting with anti-
phosphotyrosine or anti-p130^{cas} antibodies as described
above.

25   In each case, the D199A mutant PTP-PEST protein
precipitated a single broad phosphotyrosine band with an
apparent molecular weight between 120 and 150 kDa in
different cell lines, whereas the affinity matrix alone
failed to precipitate any phosphotyrosine-containing
30   protein. Immunoblotting of the precipitates with a p130^{cas}
antibody revealed that the protein precipitated from all
cell lysates corresponded to p130^{cas}; the observed
molecular weight variation between different cell lines
presumably reflects either species differences in the
35   molecular weight of p130^{cas} or expression of different

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alternatively spliced forms (Sakai et al., EMBO J. 13:3748-3756 (1994)).

The relative abundance of tyrosine phosphorylated p130^{cas} in the PTP-PEST precipitates appeared to correlate
5   approximately with the abundance of p130^{cas} protein in the lysates (data not shown). Surprisingly, regardless of the abundance of tyrosine phosphorylated p130^{cas} in the lysates, p130^{cas} was invariably the only phosphotyrosine-containing protein in the precipitates, even in 293 cell
10 lysates which contained very little p130^{cas} protein but which displayed a wide variety of other abundantly tyrosine phosphorylated proteins. Similarly, when lysates of pervanadate-treated 293 cells (containing tyrosine phosphorylated p130^{cas} in amounts which are undetectable by
15 anti-phosphotyrosine immunoblotting of the lysate) were incubated with active PTP-PEST, no visible dephosphorylation of any phosphotyrosine band occurred (Garton and Tonks, unpublished data). These results indicate that the affinity of PTP-PEST for p130^{cas} is substantially greater than for
20 any other substrate present, and further emphasizes the remarkable substrate selectivity of PTP-PEST for p130^{cas}.

Vanadate Inhibition of Tyrosine Phosphorylated p130^{cas}
Association with Mutant PTP-PEST

A consistent observation of this work was that, in
25 contrast to the inactive mutant PTP-PEST, the wild type enzyme failed to associate in a stable complex with tyrosine phosphorylated p130^{cas}, suggesting that the observed association is active site-directed. In order to investigate this possibility, mutant PTP-PEST (D199A) was
30 incubated with the PTP inhibitor vanadate at various concentrations prior to addition of pervanadate-treated HeLa cell lysate. The extent of association of p130^{cas} with PTP-PEST was then analyzed. PTP-PEST affinity matrix, comprising full length PTP-PEST (D199A) bound to covalently

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coupled protein A-Sepharose/antibody (AG25) beads, was
incubated for 10 minutes on ice in the presence of varying
concentrations of sodium orthovanadate. The samples were
then incubated with aliquots of pervanadate-treated HeLa

5   cell lysate; associated proteins were analyzed by SDS-PAGE
and immunoblotting with anti-phosphotyrosine or anti-
p130^{cas} antibodies. The activity of wild type PTP-PEST was
also determined under the same conditions, using tyrosine
phosphorylated ^{32}P-labelled RCM-lysozyme as substrate.

10   The association was found to be potently disrupted by
vanadate, with a concentration-dependence similar to that of
vanadate inhibition of wild type PTP-PEST, and complete
disruption being observed at 10 mM vanadate. Since PTP
inhibition by vanadate presumably results from a direct

15   interaction of vanadate with the active site cysteine
residue of the enzyme (Denu et al., Proc. Natl. Acad. Sci.
USA 93:2493-2498 (1996)), this result supports the
hypothesis that the stable association of mutant PTP-PEST
with tyrosine phosphorylated p130^{cas} is mediated by direct

20   interactions between active site residues within PTP-PEST,
in particular the active site cysteine residue, and
phosphotyrosine moieties within p130^{cas}.

Association of Endogenous p130^{cas} with Transfected Mutant
PTP-PEST in COS Cells

25   The work described above strongly suggests that
p130^{cas} represents a potential physiologically significant
substrate for PTP-PEST. In order to assess whether PTP-PEST
interacts with p130^{cas} in intact cells, COS cells were
transfected with plasmids encoding wild type or mutant forms

30   of PTP-PEST (D199A or C215S). The cells were treated with
pervanadate 30 minutes prior to lysis, PTP-PEST proteins
were immunoprecipitated, and associated tyrosine
phosphorylated proteins were analyzed by anti-
phosphotyrosine immunoblotting of the resultant

precipitates. Lysates were also incubated with covalently
coupled protein A-Sepharose/anti-PTP-PEST (AG25) beads and
associated proteins were analyzed by SDS-PAGE and
immunoblotting with anti-phosphotyrosine antibody.

Under these conditions, the phosphotyrosine-containing
band corresponding to p130^{cas} was again unique in its
ability to associate with the C231S PTP-PEST protein,
indicating that p130^{cas} can be specifically selected by
PTP-PEST as a substrate in an intracellular context in the
presence of a large number of alternative possible
substrates. Neither the wild type nor the D199A form of
PTP-PEST was capable of a stable interaction with tyrosine
phosphorylated p130^{cas} in pervanadate-treated COS cells.

The binding of both wild type and D199A PTP PEST to
tyrosine phosphorylated p130^{cas} under these conditions is
most likely prohibited by the presence of pervanadate bound
to the active site cysteine residue of PTP-PEST (Denu et
al., Proc. Natl. Acad. Sci. USA 93:2493-2498 (1996)), which
effectively excludes the binding of phosphotyrosine residues
of p130^{cas}. The ability of the C231S mutant PTP-PEST to
associate in a stable complex with p130^{cas} in the presence
of pervanadate suggests that this mutant protein is largely
unaffected by pervanadate, indicating that the normal mode
of inhibition of PTPs by vanadate ions depends critically on
direct interactions between vanadate and the thiolate anion
of the PTP active-site cysteine residue. These observations
therefore lend further support to the existence of an
exclusive interaction between PTP-PEST and p130^{cas} which
appears to be entirely active site-directed, and therefore
reflects the genuine, inherent, highly restricted substrate
preference of PTP-PEST for p130^{cas}.

Results described herein implicate p130^{cas} as a
physiologically relevant substrate for PTP-PEST.
Furthermore, the observed stringency and exclusivity of the
interaction between PTP-PEST and p130^{cas} in a wide variety

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of cell lines suggest that p130^{cas} may be a unique high
affinity substrate for PTP-PEST, although the possibility
that other significant PTP-PEST substrates may exist cannot
be excluded at present. In particular, it is unclear
5   whether pervanadate-treated cells display a complete
spectrum of all possible tyrosine phosphorylated proteins;
in fact this appears unlikely since pervanadate treatment
presumably results only in an increase in tyrosine
phosphorylation of proteins which are to some extent
10   constitutively phosphorylated, but which are normally
rapidly dephosphorylated, within the cell. Potential
substrates lacking from pervanadate-treated cells therefore
presumably include substrates of protein tyrosine kinases
(PTKs) which are normally present in an inactive state, such
15   as ligand-stimulated receptor PTKs, and the recently
described calcium regulated kinase PYK2 (Lev et al., Nature
376: 737-745 (1995)). Regardless of these considerations,
the ability of PTP-PEST to select p130^{cas} exclusively as a
substrate from lysates of several different cell lines,
20   containing a combined total of at least one hundred
different potential substrates (many of which presumably
contain multiple sites of phosphorylation), clearly
demonstrates that the substrate specificity of PTP-PEST is
highly restricted.
25   Many intracellular PTPs are limited in their substrate
availability due to strict confinement within a particular
subcellular location; examples include PTP1B, which is
localized to the cytoplasmic face of the endoplasmic
reticulum (Frangioni et al., Cell 68:545-560 (1992)), and
30   TCPTP which is either nuclear (Tillmann et al., Mol. Cell.
Biol. 14:3030-3040 (1994)) or localized to the endoplasmic
reticulum, depending upon which alternative spliced form is
expressed (Lorenzen et al., J. Cell Biol. 131:631-643
(1995)). Alternatively, certain PTPs appear to be highly
35   regulated, requiring activation before appreciable activity

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can be demonstrated. For example, the SH2 domain-containing
PTPs, SHP1 and SHP2, display relatively low activity in
vitro, but can be considerably activated by several
mechanisms including C-terminal truncation (Zhao et al., J.
5   Biol. Chem. 268:2816-2820 (1993)), addition of certain
phospholipids (Zhao et al., Proc. Natl. Acad. Sci. USA
90:4251-4255 (1993)), or SH2 domain-mediated binding of
appropriate phosphotyrosine-containing peptides (Lechleider
et al., J. Biol. Chem. 268:21478-21481 (1993)).

10   However, PTP-PEST exhibits high specific activity in
vitro (35,000 U/mg), and is a predominantly (90-95%) soluble
PTP within cells (Garton and Tonks, unpublished data); in
principle, therefore, it may act potently on any substrate
accessible to the cytoplasm. This accessibility may partly
15 underlie the necessity for PTP-PEST to possess an inherently
constrained substrate specificity. The demonstration that
mutant PTP-PEST is capable of exclusively associating with
p130^{cas} in an intracellular context in the presence of many
other tyrosine phosphorylated proteins, is an indication
20 that the narrow substrate specificity of the enzyme may
result in PTP-PEST having a negligible influence on the
phosphorylation state of the majority of tyrosine
phosphorylated proteins within the cell, even though those
substrates are largely accessible to PTP-PEST.

25   The role of p130^{cas} in cellular transformation by the
v-crk and v-src oncogenes is unclear, although there is a
general correlation between the level of tyrosine
phosphorylation of p130^{cas} and the degree of transformation
in cells expressing different forms of crk or src (Kanner et
30 al., EMBO J. 10:1689-1698 (1991); Mayer and Hanafusa, J.
Virol. 64:3581-3589 (1990)). Furthermore, enhanced tyrosine
phosphorylation of p130^{cas} has also been observed in cells
transformed by c-Ha-ras and by ornithine decarboxylase
overexpression (Auvinen et al., Mol. Cell. Biol. 15:6513-
35 6525 (1995)). Expression of antisense cDNA encoding

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p130^{cas} in these cells results in a partial reversion of
the transformed phenotype. These observations suggest that
aberrant tyrosine phosphorylation of p130^{cas} is a common
feature of cells transformed by several disparate mechanisms
5   and that p130^{cas} may be required for full manifestation of
the transformed state. Dephosphorylation of p130^{cas} by
PTP-PEST is therefore a potentially important regulatory
mechanism for counteracting the transforming effects of
various oncogenes.

10   Tyrosine phosphorylation of p130^{cas} has been observed
in fibroblasts following integrin-mediated cell adhesion to
extracellular matrix proteins (Nojima et al., J. Biol. Chem.
270:15398-15402 (1995); Petch et al., J. Cell Science
108:1371-1379 (1995); Vuori and Ruoslahti, J. Biol. Chem.
15 270:22259-22262 (1995)). Under these conditions, using an
antibody (4F4) that predominantly recognizes tyrosine
phosphorylated p130^{cas} (Kanner et al., EMBO J. 10:1689-1698
(1991); Petch et al., J. Cell Science 108:1371-1379 (1995))
it was shown that phosphorylated p130^{cas} is localized to
20 focal adhesions (Petch et al., J. Cell Science 108:1371-1379
(1995)), whereas fractionation studies have demonstrated
that the normal cellular location of the majority of non-
phosphorylated p130^{cas} is the cytosol (Sakai et al., EMBO
J. 13:3748-3756 (1994)). Furthermore, in crk-transformed
25 fibroblasts, tyrosine phosphorylated p130^{cas} is detected
only in insoluble fractions (Sakai et al., EMBO J. 13:3748-
3756 (1994)), suggesting that both cell adhesion- and
transformation-mediated phosphorylation of p130^{cas} is
associated with redistribution of the protein from the
30 cytosol to focal adhesions.

        It is plausible that the redistribution of tyrosine
phosphorylated p130^{cas} may be driven by its association
with FAK, which is constitutively associated with focal
adhesions due to its C-terminal focal adhesion targeting
35 domain (Hildebrand et al., J. Cell Biol. 123:993-1005

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(1993); Schaller et al., Proc. Natl. Acad. Sci. USA 89:5192-5196 (1992)). The sequestration of tyrosine phosphorylated p130^{cas} in focal adhesions both in transformed cells, and following integrin-mediated cell adhesion, strongly suggests

5   a role for p130^{cas} in signalling events in this region of the cell. One consequence of the redistribution of tyrosine phosphorylated p130^{cas} is likely to be that, in addition to localizing p130^{cas} to a region of the cell containing abundant protein tyrosine kinase activity, the

10 phosphorylated protein will be relatively inaccessible to the cytosolic phosphatase PTP-PEST. This raises the possibility that the role of PTP-PEST in dephosphorylating p130^{cas} may be to prevent inappropriate tyrosine phosphorylation of the cytosolic pool of p130^{cas}, thus

15 preventing formation of signalling complexes assembled around tyrosine phosphorylated p130^{cas} in inappropriate cellular locations.

Several mitogenic factors potently stimulate tyrosine phosphorylation of p130^{cas}. These include agents acting

20 through heterotrimeric G protein-coupled receptors such as lysophosphatidic acid (Seufferlein and Rozengurt, J. Biol. Chem. 269:9345-9351 (1994)), bradykinin (Leeb-Lundberg et al., J. Biol. Chem. 269: 24328-24344 (1994)), and bombesin (Zachary et al., J. Biol. Chem. 267:19031-19034 (1992)), as

25 well as growth factors that activate receptor tyrosine kinases, namely PDGF (Rankin and Rozengurt, J. Biol. Chem. 269:704-710 (1994)), EGF and NGF (Ribon and Saltiel, J. Biol. Chem. 271:7375-7380 (1996)). These observations suggest roles for p130^{cas} in regulation of mitogenic

30 signalling pathways, presumably involving assembly of signalling complexes based on tyrosine phosphorylated p130^{cas}. The identities of the proteins involved in these complexes are not established, but are likely to include SH2 domain containing adaptor proteins such as crk (Ribon and

35 Saltiel, J. Biol. Chem. 271:7375-7380 (1996)), and its

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associated proteins (Feller et al., Oncogene 10:1465-1473
(1995); Hasegawa et al., Mol. Cell. Biol. 16:1770-1776
(1996); Knudsen et al., J. Biol. Chem. 269:32781-32787
(1994); Matsuda et al., Mol. Cell. Biol. 14: 5495-5500
5   (1994); Tanaka et al., Proc. Natl. Acad. Sci. USA 91:3443-
3447 (1994)). Therefore tyrosine phosphorylation and
dephosphorylation of p130^{cas} potentially plays a central
role in regulating the formation of such complexes, thereby
influencing downstream events in mitogenic signalling.

10   Equivalents
         Those skilled in the art will recognize, or be able to
ascertain, using no more than routine experimentation, many
equivalents to the specific embodiments of the invention
described herein. Such equivalents are intended to be
15   encompassed by the following claims:

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CLAIMS

We claim:

1.   A protein tyrosine phosphatase wherein the invariant
     aspartate residue is replaced with an amino acid (e.g.
     alanine) which:
     a)   does not cause significant alteration of the Km
          of the enzyme but which results in a reduction in
          Kcat to less than 1 per minute; or
     b)   is selected from the group consisting of:
          alanine, valine, leucine, isoleucine, proline,
          phenylalanine, tryptophan, methionine, glycine,
          serine, threonine, cysteine, tyrosine, glutamine,
          lysine, arginine and histidine, and wherein the
          phosphatase binds to a tyrosine phosphorylated
          substrate and is catalytically attenuated.

2.   A protein tyrosine phosphatase according to claim 1(a)
     which is selected from the group consisting of: PTP1B
     (and wherein for example the invariant aspartate
     residue is located at position 181), PTP-PEST (and
     wherein for example the invariant aspartate residue is
     located at position 199), PTPτ,MKP-1, DEP-1,
     PTPμ,PTPX1, PTPX10 and PTPH1.

3.   A protein tyrosine phosphatase according to claim 1(b)
     which is a PTP-PEST phosphatase in which the amino
     acid at position 231 is replaced with a serine
     residue.

4.   A method of identifying a tyrosine phosphorylated
     protein which is a substrate of a protein tyrosine
     phosphatase, comprising the steps of:
     a)   combining at least one tyrosine phosphorylated
          protein with at least one protein tyrosine

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phosphatase in which the invariant aspartate residue is replaced with an amino acid (e.g. alanine) which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, under conditions appropriate for formation of a complex between the tyrosine phosphorylated protein and the protein tyrosine phosphatase, thereby producing a combination; and

b) determining the presence of absence of a complex in the combination

wherein the presence of a complex in the combination indicates that the tyrosine phosphorylated protein is a substrate of the protein tyrosine phosphatase with which it forms a complex.

5.   A method according to claim 4, wherein the protein tyrosine phosphatase is as defined in any one of claims 1-3.

6.   A method according to claim 4 or claim 5 wherein the tyrosine phosphorylated protein is selected from the group consisting of: $p130^{cas}$, the EGF receptor, p210 bcr:abl, MAP kinase and the insulin receptor.

7.   A kit for identifying a tyrosine phosphorylated protein substrate of a protein tyrosine phosphatase comprising:

a)   at least one protein tyrosine phosphatase in which the invariant aspartate residue is replaced with an amino acid (e.g. alanine) which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and

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        b)   ancillary reagents suitable for use in detecting
                the presence or absence of a complex between the
                protein tyrosine phosphatase and a tyrosine
                phosphorylated protein,
5   wherein for example the protein tyrosine phosphatase
        is as defined in any one of claims 1-3.

    8. A method of identifying an agent which alters the
        interaction between a protein tyrosine phosphatase and
        a tyrosine phosphatase, comprising the steps of:
10   a)   identifying a tyrosine phosphorylated protein
                which is a substrate of a protein tyrosine
                phosphatase;
        b)   combining the tyrosine phosphorylated protein and
                the protein tyrosine phosphatase and an agent to
15   be tested under conditions suitable for
                interaction between the tyrosine phosphorylated
                protein and the protein tyrosine phosphatase,
                thereby forming a combination;
        c)   determining the amount of enzymatic activity in
20   the combination; and
        d)   comparing the amount of enzymatic activity
                determined in (c) with the amount of enzymatic
                activity in the absence of the agent to be
                tested, under conditions suitable for interaction
25   between the tyrosine phosphorylated protein and
                the protein tyrosine phosphatase,
        wherein a difference in the enzymatic activity
        indicates that the agent alters the interaction
        between the protein tyrosine phosphatase and the
30   tyrosine phosphorylated protein.

    9. A method of identifying an agent which alters the
        interaction between a protein tyrosine phosphatase and
        a tyrosine phosphorylated protein which is a substrate

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of the protein tyrosine phosphatase, comprising the
steps of:

a)   identifying a tyrosine phosphorylated protein
which is a substrate of a protein tyrosine
phosphatase;

b)   combining the tyrosine phosphorylated protein, a
protein tyrosine phosphatase in which the
invariant aspartate residue is replaced with an
amino acid (e.g. alanine) which does not cause
significant alteration of the Km of the enzyme
but which results in a reduction in Kcat to less
than 1 per minute, and an agent to be tested,
under conditions suitable for interaction between
the tyrosine phosphorylated protein and the
protein tyrosine phosphatase, thereby forming a
combination;

c)   determining the extent of binding between the
tyrosine phosphorylated protein and the protein
tyrosine phosphatase in the combination; and

d)   comparing the extent of binding determined in (c)
with the extent of binding in the absence of the
agent to be tested, under conditions suitable for
interaction between the tyrosine phosphorylated
protein and the protein tyrosine phosphatase,

wherein a difference in the extent of binding
indicates that the agent alters the interaction
between the protein tyrosine phosphatase and the
tyrosine phosphatase and the tyrosine phosphorylated
protein.

10.   A method according to claim 8 or claim 9 wherein if
the amount of enzymatic activity or the extent of
binding, respectively, is:

a)   greater in the presence of the agent to be tested
than in the absence of the agent, then the agent

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enhances the interaction between the protein
tyrosine phosphatase and the tyrosine
phosphorylated protein; or

b)   less in the presence of the agent to be tested
5   than in the absence of the agent, then the agent
       inhibits the interaction between the protein
       tyrosine phosphatase and the tyrosine
       phosphorylated protein.

11. A protein tyrosine phosphatase (e.g. as defined in any
10   one of claims 1-3) for use in therapy, prophylaxis or
     diagnosis, for example in:
     a)   the treatment of conditions in which a reduction
          in the activity of a tyrosine phosphorylated
          protein is indicted; and/or
15   b)   reducing the activity of a tyrosine
          phosphorylated protein; and/or
     c)   a method of reducing the activity of a tyrosine
          phosphorylated protein, comprising administering
          to a mammal (e.g. a human) a protein tyrosine
20   phosphatase in which the invariant aspartate
          residue is replaced with an amino acid which does
          not cause significant alteration of the Km of the
          enzyme but which results in a reduction in Kcat
          to less than 1 per minute, whereby formation of
25   the complex reduces the activity of the tyrosine
          phosphorylated protein; and/or
     d)   the treatment of conditions in which a reduction
          in the transforming effects of oncogenes
          associated with p130^{cas} phosphorylation is
30   indicated; and/or
     e)   reducing the transforming effects of oncogenes
          associated with p130^{cas} phosphorylation; and/or
     f)   a method of reducing the transforming effects of
          oncogenes associated with p130^{cas} phosphorylation

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comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase which is PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, whereby the PTP binds to and/or dephosphorylates p130^{cas} and reducing the transforming effects of oncogenes associated with p130^{cas} phosphorylation; and/or

g) the treatment, therapy, diagnosis or prophylaxis of cancer (for example cancers associated with p130^{cas} phosphorylation); and/or

h) the treatment of conditions associated with oncogneic activity (e.g. with v-crk, v-src and/or c-Ha-ras activity); and/or

i) the treatment of conditions in which a reduction in the formation of signalling complexes associated with p130^{cas} is indicted; and/or

j) reducing the formation of signalling complexes associated with p130^{cas}; and/or

k) a method of reducing the formation of signalling complexes associated with p130^{cas} comprising administering to a mammal (e.g. a human) a protein tyrosine phosphatase which is PTP-PEST or PTP-PEST in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, whereby the PTP binds to and/or dephosphorylates p130^{cas} and reducing the formation of signalling complexes associated with p130^{cas}, and/or

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l)   the prevention of the induction of mitogenic
      pathways;

m)   the treatment of conditions in which the
      prevention of the induction of mitogenic pathways

5   is indicated;

n)   the treatment of conditions in which a reduction
      in the cytotoxic effects associated with protein
      tyrosine phosphatase administration or over-
      expression is indicated; and/or

10   o)   reducing the cytotoxic effects associated with
      protein tyrosine phosphatase administration or
      over-expression; and/or

p)   a method for reducing the cytotoxic effects
      associated with protein tyrosine phosphatase

15   administration or over-expression comprising
      administering to a mammal (e.g. a human) a
      protein tyrosine phosphatase in which the
      invariant aspartate residue is replaced with an
      amino acid which does not cause significant

20   alteration of the Km of the enzyme but which
      results in a reduction in Kcat to less than 1 per
      minute, in place of a corresponding wild type
      protein tyrosine phosphatase; and/or

q)   in a method according to any one of claims 13-20.

25 12. Use of a protein tyrosine phosphatase for the
      manufacture of a medicament for treatment, prophylaxis
      or diagnosis (for example for use in the treatments
      defined in claim 11).

13. A method of reducing the activity of a tyrosine
30   phosphorylated protein, comprising administering to a
      mammal (e.g. a human) a protein tyrosine phosphatase
      in which the invariant aspartate residue is replaced
      with an amino acid which does not cause significant

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alteration of the Km of the enzyme but which results
in a reduction in Kcat to less than 1 per minute,
whereby formation of the complex reduces the activity
of the tyrosine phosphorylated protein.

5   14.   A method according to claim 13, wherein the tyrosine
phosphorylated protein is selected from the group
consisting of: p130^{cas}, the EGF receptor, p210 bcr:abl,
MAP kinase and the insulin receptor.

15.   a method according to claim 13, wherein the protein
10   tyrosine phosphatase is selected from the group
consisting of: PTP1B, PTP-PEST, PTPτ, MKP-1, DEP-1,
PTPτ, PTPX1, PTPX10 AND PTPH1.

16.   A method of reducing the transforming effects of
oncogenes associated with p130^{cas} phosphorylation
15   comprising of administering to a mammal (e.g. a human)
a protein tyrosine phosphatase which is PTP-PEST or
PTP-PEST in which the invariant aspartate residue is
replaced with an amino acid which does not cause
significant alteration of the Km of the enzyme but
20   which results in a reduction in Kcat to less than 1
per minute, whereby the PTP binds to and/or
dephosphorylates p 130^{cas}, thereby negatively regulating
the downstream effects of p130^{cas} and reducing the
transforming effects of oncogenes associated with
25   p130^{cas} phosphorylation.

17.   A method according to claim 16, wherein the oncogene
is selected from the group consisting of: v-crk, v-src
and c-Ha-ras.

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18. A method of reducing formation of signalling complexes
     associated with p130^{cas} comprising administering to a
     mammal (e.g. a human) a protein tyrosine phosphatase
     which is PTP-PEST OR PTP-PEST in which the invariant
5   aspartate residue is replaced with an amino acid which
     does not cause significant alteration of the Km of the
     enzyme but which results in a reduction in Kcat to
     less than 1 per minute, whereby the PTP binds to
     and/or dephosphorylates p130^{cas}, thereby negatively
10   regulating the downstream effects of p130^{cas} and
     reducing the formation of signalling complexes
     associated with p130^{cas}.

19. A method according to claim 18, which prevents the
     induction of mitogenic pathways.

15 20. A method of reducing cytotoxic effects associated with
     protein tyrosine phosphatase administration or over
     expression, comprising administering to a mammal (e.g.
     a human) a protein tyrosine phosphatase in which the
     invariant aspartate residue is replaced with an amino
20   acid which does not cause significant alteration of
     the Km of the enzyme but which results in a reduction
     in Kcat to less than 1 per minute, in place of a
     corresponding wild type protein tyrosine phosphatase.

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                          70        80         90         100         110          120        130

Hum_PTP1B              DyINASLI          KMEEAQRSYILTqGPLPNTCGHFWEMVWE   QKSRGVVMLNRVM
Hum_TCPTP             DyINASLV           DIEEAQRSYILTqGPLPNTCCHFWLMVWQ   QKTKAVVMLNRIV
Hum_PTP_xi_D1        DGKLTDyINANYV   DGYN RPKAYIAAgGPLKSTAEDFWRMIWE      HNVEVIVMITNLV
Hum_PTP_zeia_D1      DGKLTDyINANYV   DGYN RPKAYIAAgGPLKSTAEDFWRMIWE      HNVEVIVMITNLV
Hum_PTP_gamma_D     DSKHSDyINANYV    DGYN KAKAYIATgGPLKSTFEDFWRMIWE      QNTGIIVMITNLV
Dros_PTP99A_D1      KKNL DyINANFI    DGYQ KGHAFIGTgGPLPDTFDCFWRMIWE      QRVAIIVMITNLV
Hum_LCA_D1           PGS DyINANYI    DGYR KQNAYIATgGPLPETMGDFWRMVWE      QRTATVVMTRLE
Hum_PTP_mu_D1        TNS DyINGNYI    DGYH RPNHYIATgGPMQETIYDFWRMVWH      ENTASIIMVTNLV
Hum_PTP_alpha_D1     PDS DyINASFI    NGYQ EKNKFIAAgGPKEETVNDFWRMIWE      QNTATIVMTNLK
Hum_PTP_opsilon_D    PCS DyINASYI    DGYK EKNKFIAAgGPKQETVNDFWRMIWE      QKSATIVMLTNLK
Mouso_CD45_D1        AGS TYINASYI    DGFK EPRKYIAAgGPRDETVDDFWRMIWE      QKATVIVMVTRCE
Hum_SH.PTP2          EPV SDyINANIIMPEFETKCNNSK PKKSYIATgGCLQNTVNDFWRMVFQ ENSRVIVMTTKEV
Hum_SH.PTP1          IPG SDyINANYIKNQLL GPDE NAKTYIASgGCLEATVNDFWQMAWQ   ENSRVIVMTTREV
Hum_PTP_bola         PCS DyINASYI    PGNN FRREYIVTgGPLPGTKDDFWKMVWE      QNVHNIVMVTQCV
Dros_PTP10D          EGS DyINANYV    PGHN SPREFIVTgGPLHSTRDDFWRMCWE      SNSRAIVMLTRCF
Hum_SAP.1            PGS DyINASFM    PGLW SPQEFIATgGPLPQTVGDFWRLVWE      QQSHTLVMLTNCM
Ral_PTP_STEP         DPL SSyINANYI   RGYNGEEKVVIATgGPIVSTVVDFWRMVWQ      ERTPIIVMITNIE
Dros_PTP69A_D1       QTT DyINANFV    IGYK ERKKFICAgGPMESTIDDFWRMIWE      QHLEIIVILTNLE
Hum_MEG2             TQT DyINASFM    DGYK QKNAYIGTgGPLENTYRDFWLMVWE      QKVLVIVMTTRFE
Hum_PTP.PEST         QDS DyINANFI    KGVY GPKAYVATgGPLANTVIDFWRMVWE      YNVVIVMACREF
Hum_PTPH1            INA SYVNMEI     PAAN LVNKYIATgGPLPHTCAQFWQVVWD      QKLSLIVMLTTLT
Dici_PTP1            EGS DyINANYI    DGAY   PKQFICTgGPLPNTIADFWRMVWE     NRCRIIVMLSRES
Fiss_Yeast_pyp1      EL  DyINASFI         KTETSNYIACqGSISRSISDFWHMVWDNVENIGTFVMLGSLF
Fiss_Yeast_pyp2      S   DyINASHI         DVGNKKYIACqACQAPKPGTLLDFWEMVWHNSGTNGVIVMLTNLY
Hum_PTP_xi_D2       DGKLTDyINANYV    DGYN RPKAYIAAgGPLKSTAEDFWRMIWE      HNVEVIVMITNLV
Hum_LCA_D2           EGS DyINASFL    DGYR QQKAYIATgGPLAESTEDFWRMLWE      HNSTIIVMLTKLR
Hum_PTP_alpha_D2     ENT DyVNASFI    DGYR QKDSYIASqGPLLHTIEDFWRMIWE      WKSCSIVMLTELE
Hum_PTP_opsilon_D2   EYT DyINASFI    DGYR QKDYFIATgGPLAHTVEDFWRMIWE      WKSHTIVMLTEVQ
Hum_PTP_mu_D2        SS NYINAALM     DSYK QPSAFIVTqHPLPNTVKDFWRLVLD      YHCTSVVMLNDVD
Mouse_CD45_D2        SEETSKYINASFV   MSYW KPEMMIAAqGPLKETIGDFWQMIFQ      RKVKVIVMLTELV
Dros_PTP69A_D2       ENS TYINASFI    EGYD NSETFIIAqDPFENTIGDFWRMISE      QSVTTLVMISEIG
Hum_PTP_zeia_D2      GT  DyINASYI    MGYY QSNEFIITqHPLLHTIKDFWRMIWD      HNAQLVVMIPDGQ
Hum_PTP_gamma_D2     KGT DyINASYI    MGYY RSNEFIITqHPLPHTTKDFWRMIWD      HNAQIIVMLPDNQ
Dros_PTP99A_D2      GEDGSDyINASWL    HGFR RLRDFIVTqHPMAHTIKDFWQMVWD      HNAQTVLLSSLD
Yarsinia_PTP         NyIQVG              NTRTIACqYPLQSOLESHFRMLAE        NRTPVLAVLASSS
PTP1Bseq.no.         70       80        90        100        110          120        130

                  β1      Figure 1A   β2                         α2                β3        α
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PTP1B66

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                      1          10        20        30        40        50        60
Hum_PTP1B            DFPCRVAKLPKNKN        RNRYRDVSPFDHSRIKLHQE        DN
Hum_TCPTP            DYPHRVAKFPENRN        RNRYRDVSPYDHSRV LQNA        EN
Hum_PTP_xi_D1        GITADSSNHPDNKH        KNRYINIVAYDHSRVKLAQL        AEK
Hum_PTP_zeta_D1      GITADSSNHPDNKH        KNRYINIVAYDHSRVKLAQL        AEK
Hum_PTP_gamma_D      NITAEHSNHPENKH        KNRYINILAYDHSRVKLRPL        PGK
Dros_PTP99A_D1       DLPCEHSQHPENKR        KNRYLNITAYDHSRVHLHPT        PGQ
Hum_LCA_D1           QFTWENSNLEVNKP        KNRYANVIAYDHSRVILTSI        DGV
Hum_PTP_mu_D1        SAPWDSAKKDENRM        KNRYGNIIAYDHSRVRLQTI        EGD
Hum_PTP_alpha_D1     QATCEAASKEENKE        KNRYVNILPYDHSRVHLTPV        EGV
Hum_PTP_opsilon_D    QGTFELANKEENRE        KNRYPNILPNDHSRVILSQL        DGI
Mouso_CD45_D1        KFPIKDARKPHNQN        KNRYVDILPYDYNRVELSEI        NGD
Hum_SH.PTP2          LYSRKEGQRQENKN        KNRYKNILPFDHTRVVLHDG        DPN
Hum_SH.PTP1          LHQRLEGQRPENKG        KNRYKNILPFDHSRVILQGR        DSN
Hum_PTP_bola         NQSCDIALLPENRG        KNRYNNILPYDATRVKLSNV        DDD
Dros_PTP10D          DQPCTFADLPCNRP        KNRFTNILPYDHSRFKLQPV        DDD
Hum_SAP.1            SQSQMVASASENNA        KNRYRNVLPYDWSRVPLKPI        HEE
Ral_PTP_STEP         FVDPKEYDIPGLVR        KNRYKTILPNPHSRVRLTSP        DPE
Dros_PTP69A_D1       DRTTKNSDLKENAC        KNRYPDIKAYDQTRVKLAVI        NGL
Hum_MEG2             VGTFHCSMSPGNLE        KNRYGDVPCLDQTRVKLTKR        SGH
Hum_PTP.PEST         IYPTATGEKEENVK        KNRYKDILPFDHSRVKLTLK        TPS
Hum_PTPH1            GLAITFAKLPQNLD        KNRYKDVLPYDTTRVLLQGN        EDY
Dici_PTP1            PSETSEGDKKHNTS        KNRYTNILPVNHTRVQLKKI        QDK
Fiss_yeast_pyp1      QWSTVDSLSNTSYK        KNRYTDIVPYNCTRVHLKRT        SPS
Fiss_yeast_pyp2      WCCLASSRSTSISR        KNRYTDIVPYDKTRVRLAVP        KGC
Hum_PTP_xi_D2        GITADSSNHPDNKH        KNRYINIVAYDHSRVKLAQL        AEK
Hum_LCA_D2           TSRFISANLPCNKF        KNRLVNIMPYELTRVCLQPI        RGV
Hum_PTP_alpha_D2     NDKMRTGNLPANMK        KNRVLQIIPYEFNRVIIPVK        RGE
Hum_PTP_opsilon_D2   KENMRTGNLPANMK        KARVIQIIPYDFNRVILSMK        RGQ
Hum_PTP_mu_D2        VEDCSIALLPRNHE        KNRCMDILPPDRCLPFLITI        DGE
Mouse_CD45_D2        WRTQHIGNQEENKK        KNRNSNVVPYDFNRVPLKHELEMSKESEPESDESSDDDSD
Dros_PTP69A_D2       SKSCSVGENEENNM        KNRSQEIPYDRNRVIITPL         PMR
Hum_PTP_zeta_D2      QSDYSAALKQCNRE        KNRTSSIIPVERSRVGISSL        SGE
Hum_PTP_gamma_D2     VECFSAQKECNKE         KNRNSSVVPSERARVGLAPL        PGM
Dros_PTP99A_D2       ETNLMAEQVEELKNCTPYLEQQYKNIIQFQPKDIHIASAMKQVNSIKNRGAIFPIEGSRVHLTPKP
Yarsinia_PTP         TNDPRYLQACGGEKI  LNRFRDIQCCROTAVRAD
PTP1Bseq.no.
                    30         40        50        60
                                     α1
                                    ▲▲▲
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Figure 1B

PTP1B66

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                   140        150       160          170          180                190            200

Hum_PTP1B          EKGSLKCA   QYWPQKEEKEM        IFEDTNLKLTLISEDIKSYYTVLELE                      NLTTQETREI
Hum_TCPTP          EKESVKCA   QYWPT DDQEM        LFKETGFSVKLLSEDVKSYYTVLQLE                      NINSGETRTI
Hum_PTP_xi_D1      EKGRRKCD   QYWPP ADGSE   EYGN FLVTQKSVQVLAYTVFTLRNTKIKKG                      SQKGRPSGRVV
Hum_PTP_zela_D1    EKGRRKCD   QYWP  ADGSE   EYGN FLVTQKSVQVLAYTVFTLRNTKIKKG                      SQKGRPSGRVV
Hum_PTP_gamma_D    EKGRRKCD   QYWP  TENSE   EYGN IIVTLKSTKIHACYTVFSIRNTKVKKGQKGNPKGRQNERVV      KKQCNTEKLV
Dros_PTP99A_D1     ERGRRKCD   MYWP  KDGVE   TYGV IQVKLIEEVMSTYTVLQIKHLKLKK                       KSGSSEKREL
Hum_LCA_D1         EKSRVKCD   QYWP  ARGTE   TCGL IQVTLLDTVELATYTVFALH                            KRGVHEIREI
Hum_PTP_mu_D1      EVGRVKCC   KYWP  DDTE    IYKD IKVTLIETELLAEYVIFAVE                            DMTNRKPQRLI
Hum_PTP_alpha_D1   ERKECKCA   QYWP  DQGCW   TYGN IRVSVEDVTVLVDYTVFCIQQVG                         PDGCKAPRLV
Hum_PTP_opsilon_D  ERKEEKCH   QYWP  DQGCW   TYGN IRVCVEDCVVLVDYTIFCIQPQL                         KKEKATGREV
Mouso_CD45_D1      EGNRNKCA   EYWPSMEEGTR   AFKD IVTINDHKRCPDYIILNVAH                            VGQGNTERTV
Hum_SH.PTP2        ERGKSKCV   KYWPD EYALK   EYGV MRVRNVKESAAHDYTLLKLSK                           LDNGDLIREI
Hum_SH.PTP1        ERGRNKCV   PYWPE VGMQR   AYGP YSVTNCGEHDTTEYKLLQVSP                           EEQLDAHRLI
Hum_PTP_bola       EKGRVKCD   HYWPA DQDSL   YYGD LILQMLSESVLPEWTIFKICG                           RGSEQRIL
Dros_PTP10D        EKGREKCD   QYWPN DTVPV   FYGD IKVQILNDSHYADWVMFMLC                            QVEEQKTLSV
Hum_SAP.1          EAGRVKCE   HYWPL DSQPC   THGH LRVTLVGEEVMENWTVLLLL                            RGTEERGL
Ral_PTP_STEP       EMN EKCT   EYWP  EEQV    VHDG VEITVQKVIHTEDYRLISLR                            VGEEEDRRQI
Dros_PTP69A_D1     EYNKAKCA   KYWPEKVFDTK   QFGD ILVKPAQERKTGDYIELNVSKNKAN                       NTEERQKRQV
Hum_MEG2           EGGRRKCG   QYWPLEKDSRI   RFGF LTVTNLGVENMNHYKKLEIH                            FQNESRRL
Hum_PTP.PEST       EMGRKKCE   RYWPLYGEDPI   TFAP FKISCEDEQARTDYFILLE                            NTQTGEEHTV
Hum_PTPH1          ERGRTKCH   QYWPD PPDVM   NHGG FHIQCQSEDCTIAYVSMLVT                            LTFEGETRDI
Dici_PTP1          ENCRIKCD   RYWPEQIGEGEQFSIYGNGNEVFGTYSVELVEVIQCREIITRNIR                      NANFPSVKKV
Fiss_yeast_PYP1    EAGREMCT   AYWPSNGIGDK   QVYGDYCVKQISEENVDNSRFLFEIQ                          DKPNGPPKYI
Fiss_yeast_PYP2    EAGSEKCS   QYWPDNKDHALCLEGG   LRISVOKYETFEDLKVHLFRL
Hum_PTP_xi_D2      EKGRRKCD   QYWP  ADGSE   EYGN FLVTQKSVQVLAYTVFTLRNTKIKKG                      SQKGRPSGRVV
Hum_LCA_D2         EMGREKCH   QYWP  AERSA   RYQY FVVDPMAEYNMPQYILFKVT                            DARDGQSRTI
Hum_PTP_alpha_D2   ERGQEKCA   QYWP  SDGLV   SYGD ITVELKKEEECSYTVLLVT                             NTRENKSRQI
Hum_PTP_opsilon_D2 EREQDKCY   QYWP  TEGSV   THGE ITIEIKNDTLSEAISIFLVTLNQPQ                       ARQEEQVRVV
Hum_PTP_mu_D2      PA  QLCP   QYWP  ENGVH   RHGP IQVEFVSADLEEDISFRIYNA                           ARPQDGYRMV
Mouse_CD45_D2      NGDQEVCA   QYW   GEGKQ   TYGD MEVEMKDTNRASAYTLFELR                            HSKRKEPRTV
Dros_PTP69A_D2     D  GPRKCP  RYWA  DDEVQ   YDH  ILVKYVHSESCPYYTFFYVT                            NCKIDDTLKV
Hum_PTP_zeia_D2    NMAEDEFV   YWPNKDEPINCESFKVTLMAEEHKCLSNEEKLIIFILE                             ATQDDYVLEV
Hum_PTP_gamma_D2   SLAEDEFV   YWPSREESMNCEAFTVTLISKDRLCLSNEEQIIIFILE                            ATQDDYVLEV
Dros_PTP99A_D2     D  INFA    OFWPDEATPIESDHY   RVKFLNKTNKSDYVSEVIO                             SIQDDYELTV
Yarsinia_PTP       EIANQRFGMPDYFR OSGT  YGSITVESKMTOQVGLGDGINMYTLTI                            REAGQKTISV
PTP1Bseq.no.             120       130        140        150         160

                 β5      β6           β7         β8
                                   Figure 1C
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                    210         220                 230                240      250              260         270
Hum_PTP1B           LHFHYTTwPDF G VPESPASFLNFLFKVRES GSLSPEHG                            PVVVHCSAgIGRSGTFC
Hum_TCPTP           SHFHYTTwPDF G VPESPASFLNFLFKVRES GSLNPDHG                            PAVIHCSAgIGRSGTFS
Hum_PTP_xi_D1       TQYHYTQwPDM G VPEYSLPVLTFVRKAAYA KRH   AVG                           PVVVHCSAgVGRTGTYI
Hum_PTP_zela_D1     TQYHYTQwPDM G VPEYSLPVLTFVRKAAYA KRH   AVG                           PVLVHCSAgVGRTGTYI
Hum_PTP_gamma_D     IQYHYTQwPDM G VPEYALPVLTFVRRSSAA RMP   ETG                           PIVVHCSAgVGRTGTYI
Dros_PTP99A_D1      YQYHYTNwPDH G TPDHPLPVLNFVKKSSAA NPA   EAG                           PMVVHCSAgVGRTGCFI
Hum_LCA_D1          RQFQFMAwPDH G VPEYPTPILAFLRRVKAC NPL   DAG                           PLVVHCSAgAGRTGCFI
Hum_PTP_mu_D1       RQFHFTGwPDH G VPYHATGLLGFVRQVKSK SPP   SAG                           AIVVHCSAgVGRTGTFV
Hum_PTP_alpha_D1    TQFHFTSwPDF G VPFTPIGMLKFLKKVKAC NPQ   YAG                           PIVVHCSAgVGRTGTFI
Hum_PTP_opsilon_D   SQLHFTSwPDF G VPFTPIGMLKFLKKVKTL NPV   HAG                           PIVVHCSAgVGRTGTFI
Mouso_CD45_D1       THIQFTSwPDH G VPEDPHLLLKLRRRVNAF SNF   FSG                           PVVVHCSAgIGRTGTFI
Hum_SH.PTP2         WQYHFRTwPDH G VPSDPGGVLDFLEEVHHK QESIMDAG                            PIIVHCSAgIGRTGTII
Hum_SH.PTP1         WHYQYLSwPDH G VPSEPGGVLSFLDQINQR QESLPHAG                            PTVVHCSAgVGRTGTFI
Hum_PTP_bola        RHFHYTVwPDH G VPETTQSLIQFVRTVRDY INRSPGAG                            PIVVHCSAgVGRSGTFI
Dros_PTP10D         RHFHFTTwPDF G VPNPPQTLVRFVRAFRDR ICA   EQR                           PPIVHCSAgVGRTGTLI
Hum_SAP.1           RQFHYQAwPDH G VPSSPDTLLAFWRMLRQW LDQTMEGG                            PIIVHCSAgIGRTGCFI
Ral_PTP_STEP        KHYWFTSwPDQ K TPDRAPPLLHLVREVEEAAQQEGPHCS        RG                 PILVHCSAgVGRTGTLV
Dros_PTP69A_D1      TQYHYLTwKDF M APEHPHGIIKFIRQINSVYSLQ             RG                 PIVVHCSAgIGRTGTFC
Hum_MEG2            THFQFLSwPDY G VPSSAASLIDFLRVVRNQQSLAVSNMGARSKGQCPEPPIVVHCSAgIGRTGTFC
Hum_PTP.PEST        YQFHYVNwPDH D VPSSFDSILDMISLMRKYQEHE DV                              PICIHCSAgCGRTGAIC
Hum_PTPH1           THLQYVAwPDH G IPDDSSDFLEFVNYVRSLRVDSE                                PVLVHCSAgIGRTGVLV
Dici_PTP1           TQYQYEGwPDH N IPDHTQPFRQLLHSITNRQNQIIPSSD             RNVPIIVHCSAgVGRTGTFC
Fiss_yeast_pyp1     HHYQYPNwSDC N SPENVKSMVEFLKYVNNSHGSG                                 NTIVHCSAgVGRTGTFI
Fiss_yeast_pyp2     HHFWVHTwFD  K THPDIESITGLIRCIDKVPNDG                                 PMFVHCSAgVGRTGTFI
Hum_PTP_xi_D2       TQYHYTQwPDM G VPEYSLPVLTFVRKAAYA KRH   AVG                           PVVVHCSAgVGRTGTYI
Hum_LCA_D2          RQFQFTDwPEQ G VPKTGEGFIDFIGQVHKT KEQFGQDG                            PITVHCSAgVGRTGVFI
Hum_PTP_alpha_D2    RQFHFHGwPEV G IPSDGKGMISIIAAVQKQ QQQ   SGNH                          PITVHCSAgAGRTGTFC
Hum_PTP_opsilon_D2  RQFHFHGwPEI G IPAEGKGMIDLIAAVQKQ QQQ   TGNH                          PITVHCSAgAGRTGTFI
Hum_PTP_mu_D2       QQFQFLGwPMYRD TPVSKRSFLKLIRQVDKWQEEYNGGEG                            PTVVHCLNgGGRSGTFC
Mouse_CD45_D2       YQYQCTTwKGE E LPAEPKDLVSMIQDLKQKLPKASPEGMKYH             KHASILVHCRDgSQQTGLFC
Dros_PTP69A_D2      TQFQYNGwPTVDGEVPEVCRGIIELVDQAYNHYKNNKNSGC              RSPLTVHCSLgTDRSSIFV
Hum_PTP_zeia_D2     RHFQCPKwPN     PDSPISKTFELISVIKEEAANR          DG                 PMIVHDEHgGVTAGTFC
Hum_PTP_gamma_D2    RHFQCPKwPN     PDAPISSTFELINVIKEEALTR          DG                 PTIVHDEYgAVSAGMLC
Dros_PTP99A_D2      KMLHCPSwPEM    SNPNSIYDFIVDVHERCNDY           RNG                PIVIVDRYgGAOACTFC
Yarsinia_PTP        PVVHVGNwPDQTAVSSEVTKALASLVDQTAETKRMYESKGSSAVADDSKLRPVIHCRAgVGRTAOLI
PTP1Bseq.no.

        β9                                                                β10
       180           190           200                                   210          220
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Figure 1D

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                     280         290         300         310         320         330         340
Hum_PTP1B            LADTCLLLMDKR  KDPSSVDI KKVLLEMRKFRMG LIQTADQLRFSYLAVIEGAKFIMGD
Hum_TCPTP            LVDTCLVLMEKG  DD INI   KQVLLNMRKYRMG LIQTPDQLRFSYMAIIEGAKCIKGDSS
Hum_PTP_xi_D1       VLDSMLQQIQHE  GT VNI   FGFLKHIRSQRNY LVQTEEQYVFIHDTLVEAILSKETEV
Hum_PTP_zela_D1     VLDSMLQQIQHE  GT VNI   FGFLKHIRSQRNY LVQTEEQYVFIHDTLVEAILSKETEV
Hum_PTP_gamma_D    VIDSMLQQIKDK  ST VNV   LGFLKHIRTQRNY LVQTEEQYIFIHDALLEAILGKETEV
Dros_PTP99A_D1     VLDAMLKQIQQK  NI VNV   FGFLRHIRAQRNF LVQTEEQYIFLHDALVEAIASGETNL
Hum_LCA_D1         VIDAMLERMKHE  KT VDI   YGHVTCMRSQRNY MVQTEDQYVFIHEALLEAATCGHTEV
Hum_PTP_mu_D1      VIDIMLDMAERE  GV VDI   YNCVRELRSRRVN MVQTEEQYVFIHDAILEACLCGDTSV
Hum_PTP_alpha_D1   VIDAMLDMMHTE  RK VDV   YGFVSRIRAQRCQ MVQTDMQYVFIYQALLEHYLYGDTEL
Hum_PTP_opsilon_D  VIDAMMAMMHAE  QK VDV   FEFVSRIRNQRPQ MVQTDMQYTFIYQALLEYYLYGDTEL
Mouso_CD45_D1      GIDAMLEGLEAE  GK VDV   YGYVVKLRRQRCL MVQVEAQYILIHQALVEYNQFGETEV
Hum_SH.PTP2        VIDILIDIIREK  GVDCDIDV PKTIQMVRSQRSG MVQTEAQYRFIYMAVQHYIETLQRRI
Hum_SH.PTP1        VIDMLMENISTK  GLDCDIDI QKTIQMVRAQRSG MVQTEAQYKFIYVAIAQFIETTKKL
Hum_PTP_bola       ALDRILQQLDSK  DS VDI   YGAVHDLRLHRVH MVQTECQYVLHQCVRDVLRARKLRS
Dros_PTP10D        TLDRILQQINTS  DY VDI   FGIVYAMRKERVW MVQTEQQYICIHQCLLAVLEGKENIVGP
Hum_SAP.1          ALDVLLRQLQSE  GL LGP   FSFVRKMRESRPL MVQTEAQYVFLHQCICGSSNSQPRPQPR
Ral_PTP_STEP       ATSICCQQLRRE  GV VDI   LKTTCQLRQDRGG MIQTCEQYQFVHHAMSLY
Dros_PTP69A_D1     ALDSLIQQLEEE  DS VSI   YNTVCDLRHQRNF LVQSLKQYIFLYRALLDTGTFGNTDI
Hum_MEG2           SLDICLAQLEEL  GT LNV   FQTVSRMRTQRAF SIQTPEQYFCYKAILEFA
Hum_PTP.PEST       AIDYTWNLLKAG  KIPEEFNV FNLIQEMRTQRHS AVQTKEQYELVHRAIAQLFEKQLQLY
Hum_PTPH1          TMETAMCLTERN  LP IYP   LDIVRKMRDQRAM MVQTSSQYKFVCEAILRVY
Dici_PTP1          TAVIMKKLDHYFKQLDYNSRIDFNL FSIVLKLREQRPG MVQQLEQYLFCYKTILDEIYHRLNC
Fiss_yeast_pyp1    VLDTILRFPESKLSGFNPSVADSSDVVFQLVDHIRKQRMK MVQTFTQFKYVYD LIDSL
Fiss_yeast_pyp2    AVDQLLQVPKNILPK TTNLEDSKDFIENCVNSLRSQRMK MVQNFEQEKFLYD VVDYL
Hum_PTP_xi_D2      VLDSMLQQIQHE  GT VNI   FGFLKHIRSQRNY LVQTEEQYVFIHDTLVEAILSKETEV
Hum_LCA_D2         TLSIVLERMRYE  GV VDM   FQTVKTLRTQRPA MVQTEDQYQLCYRAALEYL
Hum_PTP_alpha_D2   ALSTVLERVKAE  GI LDV   FQTVKSLRLQRPH MVQTLEQYEFCYKVVQEYI
Hum_PTP_opsilon_D2 ALSNILERVKAE  GL LDV   FQAVKSLRLQRPH MVQTLEQYEFCYKVVQDFI
Hum_PTP_mu_D2      AISIVCEMLRHQ  RT VDV   FHAVKTLRNNKPN MVDLLDQYKFCYEVALEYLNSG
Mouse_CD45_D2      ALFNLLESAETE  DV VDV   FQVVKSLRKARPG VVCSYEQYQFLYDIIASIYPAQNGQV
Dros_PTP69A_D2     AMCILVQHLRLE  KC VDI   CATTRKLRSQRTG LINSYAQYEFLHRAIINY
Hum_PTP_zeia_D2    ALTTLMHQLEKE  NS VDV   YQVAKMINLMRPG VFADIEQYQFLYKVILSLVSTRQEEN
Hum_PTP_gamma_D2   ALTTLSQQLENE  NA VDV   FQVAKMINLMRPG VFTDIEQYFIYKAMLSLVSTKENGN
Dros_PTP99A_D2     AISSLAIEMEYC  ST ANV   YOYAKLYHNKRPG WTSSEDIRVIYN ILSFLPGNLNLLKR
Yarsinia_PTP       GAMCMNDSRNSO  LSV EDMVSOMRVORNG MVQKDEOLDVLIK LAE
PTP1Bseq.no.       230       240       250       260       270       280
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Figure 1E

α4   α5   α6

PTP1B66

6/6

Vmax and Km of 37kDa-PTP1B Mutants Toward RCML

Enzyme	Vmax (nmol/min/mg)	Km (nM)	Kcat (min^{-1})
wild type	60200	102	2244
Tyr 46 → S	4120	1700	154
→ L	4160	1700	155
Glu 115 → A	5700	45	212
→ D	5900	20	220
Lys 116 → A	68600	150	2557
Lys 120 → A	19000	80	708
Asp 181 → A	0.61	≤126	0.023
→ E	97	10	3.6
His 214 → A	700	20	26
Cys 215 → S	0.026		0.00097
Arg 221 → K	11	80	0.41
→ M	3.3	1060	0.12
Gln 262 → A	720	9	27

Figure 2